NEW INTRAVESICAL TREATMENT MODALITIES FOR NON-MUSCLE INVASIVE BLADDER CANCER

Harm Christiaan Arentsen

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Colofon

New intravesical treatment modalities for non-muscle invasive bladder cancer.

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„Es siegte die Stärke, und krönet zum Lohn
die Schönheit und Weisheit mit ewiger Kron'!"
(J.E. Schikaneder, Die Zauberflöte)

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Content

Chapter 1

General introduction and outline of the thesis

General introduction

Bladder cancer is a major health problem. According to the nationwide Netherlands Cancer Registry, bladder cancer accounted for 6,149 new cases in the Netherlands in 2011, which was 5.4% of all newly diagnosed cancers that year. The age-adjusted (to the European population) incidence rate per 100,000 person-years was 45.2 in men and 11.5 in women in 2011[1]. The incidence of bladder tumours is increasing, and consequently the financial burden of its diagnosis, treatment and follow-up is rising. Bladder cancer entails a very high per-patient cost from diagnosis to death. In fact, the cost per patient with bladder cancer is the highest of all cancers [2] due to the lifelong need to monitor for and treat recurrent tumours.

The most common histological subtype of bladder cancer is urothelial carcinoma, accounting for more than 90-95% of all bladder tumours [3]. Approximately 75-85% of patients with bladder cancer presents with non-muscle invasive bladder cancer (NMIBC), i.e., Ta, T1, and carcinoma *in situ* (CIS). The remainder of patients present with tumours invading the detrusor muscle (stage T2), the perivesical tissue (T3), or the organs surrounding the bladder (T4) (figure 1) [4]. Stage Ta tumours have a papillary configuration of their exophytic part, are confined to the urothelium, and do not penetrate into the lamina propria or detrusor muscle. Stage T1 tumours originate from the urothelium but penetrate the basement membrane which separates the urothelium from the deeper layers. They invade into the lamina propria, but do not reach the detrusor muscle.

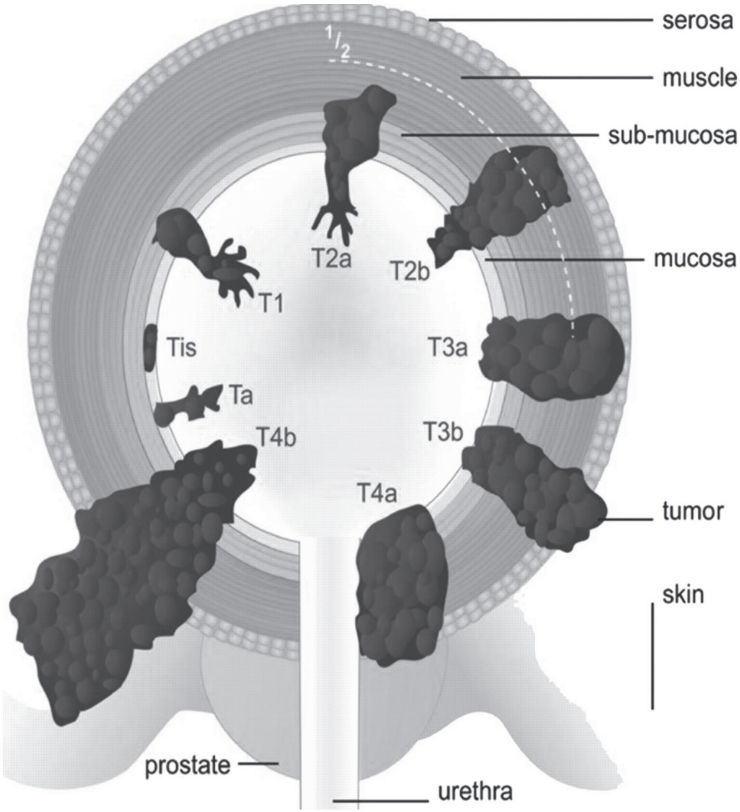

Figure 1: *TNM staging of bladder cancer [4]. Adapted from Verma et al. [37].*

Currently, two classifications are used for grading of papillary NMIBC (WHO 1973 and 2004; table 1) [5]. 1973 WHO grade 1 carcinomas have been reassigned to papillary urothelial neoplasms of low malignant potential (PUNLMP) and low-grade carcinomas in the 2004 WHO classification. Grade 2 carcinomas, which were the subject of controversy, have been eliminated in the 2004 WHO classification and have been reassigned to low-grade or high-grade carcinomas. The 2004 WHO classification contains a detailed histological description

of the various grades, which should result in less diagnostic variability among pathologists, however, the published comparisons have not clearly confirmed a better reproducibility for the WHO 2004 classification [6,7]. Several studies have compared the two classifications concerning prognostic implications with conflicting results [7-11]. Until the prognostic role of this classification has been validated by more prospective trials with sufficient follow-up, both classifications should be used, as indicated by the 2013 European Association of Urology (EAU) guideline on NMIBC as a grade A recommendation [12]. Moreover, the majority of clinical trials published so far have been performed using the 1973 WHO classification, and therefore advises in currently used guidelines are based on this version.

Table 1: *World Health Organization (WHO) grading 1973 and 2004 [5].*

1973 WHO grading
Urothelial papilloma
Grade 1: well differentiated
Grade 2: moderately differentiated
Grade 3: poorly differentiated
2004 WHO grading
Urothelial papilloma
Papillary urothelial neoplasm of low malignant potential (PUNLMP)
Low-grade papillary urothelial carcinoma
High-grade papillary urothelial carcinoma

CIS is a high-grade (anaplastic) carcinoma confined to the urothelium, but with a flat non-papillary configuration. Unlike a papillary tumour, CIS appears as reddened and velvety mucosa and is slightly elevated, although sometimes not visible. The diagnosis of CIS is based on the histology of biopsies from the bladder wall.

The standard initial therapy for Ta and T1 papillary bladder tumours is complete macroscopic transurethral resection (TURBT) including a part of the underlying muscle [13,14]. This allows for the initial staging that is critical for further management decisions. A re-resection is advised if there is any doubt about the completeness of the initial TURBT, or if there was no muscle in the specimen (with the exception of Ta grade 1 tumours and primary CIS). In all T1 and/or grade 3 tumours a restaging second TURBT should be performed 2-6 weeks after the initial TURBT [15], except for patients with CIS alone. Since there is considerable risk for recurrence and/or progression of tumours after TURBT, perioperative and/or adjuvant intravesical therapy is recommended. As NMIBC is a heterogeneous disease, intravesical therapy is risk group adapted. Risk stratification depends on clinical (number of tumours, tumour size, prior recurrence rate), and pathological (T category, CIS, grade) factors. The European Organization for Research and Treatment of Cancer Genitourinary (EORTC-GU) group has developed a scoring system and risk tables that predicts separately the risks of recurrence and progression in individual patients at different intervals after TURBT [16]. The EAU guideline [12] recommends stratification of patients into three risk groups and provides practical treatment recommendations (table 2). The stratification is based on the EORTC-GU risk tables and on the advises of the International Bladder Cancer Group [17], and is rather similar to the American Urological Association (AUA) risk group stratification [18].

Table 2: *Treatment recommendations in NMIBC according to risk group stratification.*

Risk category	Definition	Treatment recommendation
Low	Primary, solitary, Ta, G1 (low grade), < 3 cm, no CIS	One immediate instillation of chemotherapy
Intermediate	All cases not defined in the two adjacent categories	One immediate instillation of chemotherapy followed by further instillations, either chemotherapy for a maximum of 1 year or 1 year full-dose BCG
High	Any of the following: • T1 • G3 (high grade) • CIS • multiple and recurrent and large (> 3 cm) TaG1-2 tumours (all conditions must be presented)	Full-dose BCG instillations for 1-3 years or cystectomy (in highest risk tumours)

Patients at low risk for both recurrence and progression, with a primary, solitary, small (< 3 cm), low grade Ta tumour without CIS, should receive one immediate post-TURBT intravesical instillation with a chemotherapeutic agent (i.e., mitomycin C, epirubicin) [19]. No further treatment is recommended prior to recurrence.

In patients with intermediate-risk tumours (i.e., all cases between categories of low- and high-risk), the use of one immediate postoperative instillation of chemotherapy is not supported by consistent data [20], however still recommended in the EAU guideline. Adjuvant intravesical therapy is necessary but consensus regarding the optimal drug and the most appropriate scheme is lacking. Intravesical immunotherapy with bacillus Calmette-Guérin (BCG) maintenance therapy shows a better reduction of the recurrence rate, but the impact on tumour progression is uncertain [21-24]. Moreover, BCG-related side effects are more severe and more frequent as compared to chemotherapy [25,26]. In case adjuvant intravesical chemotherapy is administered, the schedule is optional although the duration of treatment should not exceed one year [27].

Patients with TaT1 high grade tumours with or without CIS and those with CIS alone are at high risk of progression. The value of one immediate instillation is at least doubtful, and, for example, certainly not supported by the most recent recommendations of the ICUD (International Consensus on Urological Diseases) on bladder cancer [28].

Adjuvant intravesical BCG maintenance therapy for at least one year is the treatment of choice [23,24]. BCG maintenance for three years reduces recurrences as compared with one year, however the benefit of the two additional years should be weighed against its additional costs, side effects, and inconvenience [29]. Immediate cystectomy should be considered in patients at highest risk of tumour progression [12,30].

Unfortunately, a substantial percentage of treated patients still experiences tumour recurrences or even progression to muscle invasive bladder cancer. This suboptimal treatment outcome can partly be attributed to low compliance with bladder cancer guideline therapy advises. For example, Witjes *et al.* [31] found a marked underuse

of guideline-recommended adjuvant intravesical therapy in patients with intermediate- and high-risk NMIBC in a retrospective chart review study. Only 50% of high-risk patients received BCG maintenance therapy as recommended by the EAU and AUA guidelines. In intermediate-risk patients, only 47% received AUA or EAU guideline-recommended adjuvant intravesical therapy. 24% of intermediate-risk patients and 9% of high-risk patients received only TURBT, with no further intravesical therapy. These findings are consistent with other studies that have found poor adherence to bladder cancer guidelines and underuse of BCG [32-36].

However, even with the use of adjuvant intravesical treatment as recommended in current guidelines, the number of recurrences and progression is still substantial. A recent large EORTC-GU study of maintenance BCG in intermediate- and high-risk TaT1 papillary bladder tumours with a median follow-up of 7.1 year showed a recurrence in 587 of 1,355 patients (43.3%). Progression to muscle invasive disease (≥ pT2) was seen in 109 (8.0%) patients and 67 (4.9%) developed distant metastases. A total of 68 patients (5.0%) died due to bladder cancer [29]. Progression rates for high-risk patients will obviously be even higher than for this mixed group of intermediate- and high-risk patients (only 41.3% of patients in this study had T1 and/or grade 3 tumours and CIS patients were excluded).

BCG is considered to have failed whenever (1) muscle-invasive tumour is detected during follow-up; (2) high-grade papillary NMIBC is present at 3 months; (3) CIS (without concomitant papillary tumour) is present at both 3 and 6 months. In patients with CIS present at 3 months, an additional BCG course can achieve a complete response in > 50% of cases; or (4) any worsening of disease occurs under BCG treatment such as higher number of recurrences, higher T stage or higher grade, or appearance of CIS, despite an initial response [12]. Patients failing on BCG immunotherapy are unlikely to respond to further BCG therapy, and there are no standard accepted alternative

bladder-preserving strategies available. This still is a highly unmet clinical need, and research into this area should be and is done. Currently, in this patient group, radical cystectomy still is indicated [12]. However, radical cystectomy is major surgery and not a suitable treatment option for a subset of patients.

In conclusion, initial therapy of NMIBC is transurethral resection, followed by intravesical chemotherapeutic or immunotherapeutic therapy. Drugs and treatment schedule are risk group adapted and described in guidelines. However, due to a low compliance to follow guideline therapy advises and even with the use of adjuvant intravesical instillations, a substantial percentage of treated patients still experiences tumour recurrences or even progression to muscle invasive bladder cancer. In patients failing on intravesical BCG there is no accepted standard alternative conservative approach. Moreover, current intravesical therapy is not without toxicity and serious local and systemic adverse effects may occur. Therefore, current intravesical treatment strategies against NMIBC are suboptimal. To reduce toxicity and to enhance efficacy improved new intravesical treatment modalities are urgently needed.

Outline of the thesis

In this thesis the preclinical testing of several potential new intravesical treatment modalities for NMIBC are described. In ***chapter 2*** the relative cell killing effect of meso-tetraphenyl chlorin disulphonate ($TPCS_{2a}$)-based photodynamic therapy is described for 4 human bladder cancer cell lines and a rat bladder cancer cell line. Secondly, enhanced cytotoxicity of bleomycin was studied with $TPCS_{2a}$-based photochemical internalization. Three commonly used anti-bladder cancer intravesical chemotherapeutic agents (i.e., epirubicin, gemcitabine and mitomycin C) were used as controls. In this unique procedure site-specific delivery of several types of membrane-im-

permeable molecules (e.g., bleomycin) to the cytosol of target cells (e.g., tumour cells) can be achieved. The technology is based on photochemical-induced release of endocytosed macromolecules from endosomes and lysosomes into the cytosol.

While *in vitro* models are useful for initial development and evaluation of therapeutic agents and modalities, adequate animal models are still essential in the preclinical development of new effective and safe therapies for many human malignancies. To enable preclinical testing of new intravesical therapeutic agents, a suitable bladder tumour model that resembles human disease is highly desirable. In ***chapter 3*** the problems associated with current *in vivo* animal bladder tumour models are discussed, focusing on the orthotopic syngeneic rat bladder tumour model. In the second part of the chapter the development of a potential new orthotopic rat bladder tumour model is described.

In ***chapter 4*** the results of an efficacy and safety study with different formulations of the relatively new drug apaziquone in the orthotopic Fischer/AY-27 rat bladder cancer model are discussed. Although the activity of intravesical apaziquone has been demonstrated in clinical marker lesion studies, to date apaziquone has not been tested in orthotopic bladder cancer models. Moreover, during clinical development of apaziquone, buffer composition of the formulation was slightly modified, in order to get a better lyophilized product. Although these modifications were not expected to affect the anti-tumour activity of apaziquone, formal evidence was lacking and it was therefore considered important to examine the activity of different formulations.

The antiproliferative effect of protodynamic therapy with *cis*-urocanic acid (*cis*-UCA) against bladder cancer cells is discussed in ***chapter 5***. Protodynamic therapy is a recently introduced concept of cancer treatment which comprises the inhibition of cancer cell

proliferation by intracellular acidification leading to apoptosis and cell death. *Cis*-UCA acts as a protodynamic drug capable of transporting protons from the mildly acidic extracellular medium into the tumour cell cytosol. Safety and anti-tumour activity of repeated intravesical *cis*-UCA administration were assessed in the orthotopic Fischer/AY-27 rat bladder urothelial carcinoma model.

The results of a preclinical study in pigs are discussed in ***chapter 6.*** R-837 is the lead compound of the imidazoquinoline family (Toll-like receptor-7 (TLR-7) agonists). Activation of TLR-7 by R-837 leads to an intracellular signaling cascade, causing a potent anti-tumour immune response. Imidazoquinolines also have potent direct biological effects on urothelial carcinoma cells by decreasing cell viability and inducing apoptosis and cytokine production. Additionally, initial results in an immune competent, orthotopic mouse model suggested anti-tumour effects *in vivo*. Therefore, imidazoquinolines may have therapeutic potential as intravesical agent for bladder cancer. To test whether R-837 might be suitable for bladder cancer therapy, TLR-7 expression in human bladder cancer and normal bladder tissue is assessed. To test the potential of R-837 for intravesical use, a pig model was used to study pharmacokinetic properties and toxicity of three different intravesical formulations with R-837.

References

1. Netherlands Cancer Registry, IKNL © february 2013
2. Botteman MF, Pashos CL, Redaelli A *et al.*
   The health economics of bladder cancer. A comprehensive review of the published literature. Pharmacoeconomics 2003;21:1315-1330
3. Ploeg M, Aben KKH, Hulsbergen-Van de Kaa CA *et al.*
   The clinical epidemiology of non-urothelial bladder cancer. J Urol 2010;183:915-920
4. Sobin LH, Gospodarowicz MK and Wittekind C, editors.
   TNM classification of malignant tumors (UICC International Union Against Cancer). ed 7. New York, NY: Wiley-Blackwell; 2009. p. 262-265
5. Epstein JI, Amin MB, Reuter VR *et al.*
   The World Health Organization International Society of Urological Pathology consensus classification of urothelial (transitional cell) neoplasms of the urinary bladder. Am J Surg Pathol 1998;22:1435-1448
6. Van Rhijn BWG, van Leenders GJLH, Ooms BCM *et al.*
   The pathologist's mean grade is constant and individualizes the prognostic value of bladder cancer grading. Eur Urol 2010;57:1052-1057
7. May M, Brookman-Amissah S, Roigas J *et al.*
   Prognostic accuracy of individual uropathologists in noninvasive urinary bladder carcinoma: A multicentre study comparing the 1973 and 2004 World Health Organisation classifications. Eur Urol 2010;57:850-858
8. Cao D, Vollmer RT, Luly J *et al.*
   Comparison of 2004 and 1973 World Health Organization grading systems and their relationship to pathologic staging for predicting long-term prognosis in patients with urothelial carcinoma. Urology 2010;76:593-599
9. Pellucchi F, Freschi M, Ibrahim B *et al.*
   Clinical reliability of the 2004 WHO histological classification

system compared with the 1973 WHO system for Ta primary bladder tumors. J Urol 2011;186:2194-2199
10. Otto W, Denzinger S, Fritsche HM *et al.*
The WHO classification of 1973 is more suitable than the WHO classification of 2004 for predicting survival in pT1 urothelial bladder cancer. BJU Int 2010;107:404-408
11. Chen Z, Ding W, Xu K *et al.*
The 1973 WHO classification is more suitable than the 2004 WHO classification for predicting prognosis in non-muscle-invasive bladder cancer. PLoS ONE 2012;7:e47199. doi:10.1371/journal.pone.0047199
12. Babjuk M, Burger M, Zigeuner R *et al.*
EAU Guidelines on non-muscle-invasive urothelial carcinoma of the bladder: Update 2013. Eur Urol 2013;64:639-653
13. Brausi M, Collette L, Kurth K *et al.*, EORTC Genito-Urinary Tract Cancer Collaborative Group.
Variability in the recurrence rate at first follow-up cystoscopy after TUR in stage Ta T1 transitional cell carcinoma of the bladder: a combined analysis of seven EORTC studies. Eur Urol 2002;41:523-31
14. Mariappan P, Zachou A and Grigor KM.
Detrusor muscle in the first, apparently complete transurethral resection of bladder tumour specimen is a surrogate marker of resection quality, predicts risk of early recurrence, and is dependent on operator experience. Eur Urol 2010;57:843-849
15. Divrik RT, Şahin AF, Yildirim Ü *et al.*
Impact of routine second transurethral resection on the long-term outcome of patients with newly diagnosed pT1 urothelial carcinoma with respect to recurrence, progression rate, and disease-specific survival: a prospective randomised clinical trial. Eur Urol 2010;58:185-190
16. Sylvester RJ, van der Meijden AP, Oosterlinck W *et al.*
Predicting recurrence and progression in individual patients with stage TaT1 bladder cancer using EORTC risk tables: a com-

bined analysis of 2596 patients from seven EORTC trials. Eur Urol 2006;49:466-477
17. Brausi M, Witjes JA, Lamm D *et al.*
A Review of Current Guidelines and Best Practice Recommendations for the Management of Nonmuscle Invasive Bladder Cancer by the International Bladder Cancer Group. J Urol 2011;186:2158-2167
18. Hall MC, Chang SS, Dalbagni G *et al.*
Guideline for the management of nonmuscle invasive bladder cancer (stages Ta, T1, and Tis): 2007 update. J Urol 2007;178:2314-2330
19. Sylvester RJ, Oosterlinck W and van der Meijden AP.
A single immediate postoperative instillation of chemotherapy decreases the risk of recurrence in patients with stage Ta T1 bladder cancer: a meta-analysis of published results of randomized clinical trials. J Urol 2004;171:2186-2190
20. Gudjonsson S, Adell L, Merdasa F *et al.*
Should all patients with non-muscle-invasive bladder cancer receive early intravesical chemotherapy after transurethral resection? The results of a prospective randomized multicentre study. Eur Urol 2009;55:773-780
21. Sylvester RJ, van der Meijden AP and, Lamm DL.
Intravesical bacillus Calmette-Guérin reduces the risk of progression in patients with superficial bladder cancer: a meta-analysis of the published results of randomized clinical trials. J Urol 2002;168:1964-1970
22. Böhle A and Bock PR.
Intravesical bacille Calmette-Guérin versus mitomycin C in superficial bladder cancer: Formal meta-analysis of comparative studies on tumor progression. Urology 2004;63:682-687
23. Malmström PU, Sylvester RJ, Crawford DE *et al.*
An individual patient data meta-analysis of the long-term outcome of randomised studies comparing intravesical mitomycin C versus Bacillus Calmette-Guérin for non-muscle-invasive bladder

cancer. Eur Urol 2009;56:247-256
24. Sylvester RJ, Brausi MA, Kirkels WJ *et al.*
Long-term efficacy results of EORTC Genito-Urinary Group randomized phase 3 study 30911 comparing intravesical instillations of epirubicin, bacillus Calmette-Guérin, and bacillus Calmette-Guérin plus isoniazid in patients with intermediate- and high-risk stage Ta T1 urothelial carcinoma of the bladder. Eur Urol 2010;57:766-773
25. Böhle A, Jocham D and Bock PR.
Intravesical bacillus Calmette-Guérin versus mitomycin C for superficial bladder cancer: a formal meta-analysis of comparative studies on recurrence and toxicity. J Urol 2003;169:90-95
26. Van der Meijden APM, Sylvester RJ, Oosterlinck W *et al.*
Maintenance bacillus Calmette-Guerin for TaT1 bladder tumors is not associated with increased toxicity: results from a European Organisation for Research and Treatment of Cancer Genito-Urinary Group phase III trial. Eur Urol 2003;44:429-434
27. Sylvester RJ, Oosterlinck W and Witjes JA.
The schedule and duration of intravesical chemotherapy in patients with non-muscle-invasive bladder cancer: a systematic review of the published results of randomized clinical trials. Eur Urol 2008;53:709-719
28. Burger M, Oosterlinck W, Konety B *et al.*
ICUD-EAU International consultation on bladder cancer 2012: Non-muscle-invasive urothelial carcinoma of the bladder. Eur Urol 2013;63:36-44
29. Oddens J, Brausi M, Sylvester R *et al.*
Final results of an EORTC-GU Cancers Group randomized study of maintenance bacillus Calmette-Guérin in intermediate- and high-risk Ta, T1 papillary carcinoma of the urinary bladder: one-third dose versus full dose and 1 year versus 3 years of maintenance. Eur Urol 2013;63:262-272
30. Denzinger S, Fritsche HM, Otto W *et al.*
Early versus deferred cystectomy for initial high-risk pT1G3

urothelial carcinoma of the bladder: do risk factors define feasibility of bladder-sparing approach? Eur Urol 2008;53:146-152
31. Witjes JA, Palou J, Soloway M *et al.*
Current clinical practice gaps in the treatment of intermediate- and high-risk non-muscleinvasive bladder cancer (NMIBC) with emphasis on the use of bacillus Calmette-Guérin (BCG): results of an international individual patient data survey (IPDS). BJU Int 2013. doi: 10.1111/bju.12012
32. Chamie K, Saigal CS, Lai J *et al.*
The Urologic Diseases in America Project. Compliance with guidelines for patients with bladder cancer: variation in the delivery of care. Cancer 2011;117: 5392-5401
33. Gontero P, Oderda M, Altieri V *et al.*
Are referral centers for non-muscle-invasive bladder cancer compliant to EAU guidelines? A report from the Vesical Antiblastic Therapy Italian Study. Urol Int 2011;86: 19-24
34. Adiyat KT, Katkoori D, Soloway CT *et al.*
"Complete transurethral resection of bladder tumor": Are the guidelines being followed? Urology 2010;75:365-369
35. Skolarus TA, Ye Z, Montgomery JS *et al.*
Use of restaging bladder tumor resection for bladder cancer among medicare beneficiaries. Urology 2011;78:1345-1350
36. Madeb R, Golijanin D, Noyes K *et al.*
Treatment of nonmuscle invading bladder cancer: Do physicians in the United States practice evidence based medicine? Cancer 2009;115:2660-2670
37. Verma S, Rajesh A, Prasad SR *et al.*
Urinary bladder cancer: Role of MR Imaging. Radiographics 2012;32:371-387

Chapter 2

The effect of photochemical internalization of bleomycin in the treatment of urothelial carcinoma of the bladder: An in vitro study

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Abstract

Objective

In this *in vitro* study, we determined whether meso-tetraphenyl chlorin disulphonate ($TPCS_{2a}$)-based photochemical delivery of bleomycin was able to potentiate the cytotoxicity of bleomycin on bladder cancer cells.

Materials and methods

The human RT4, RT112, 253J, T24, and rat AY-27 urothelial carcinoma cell lines were used. Cells were seeded in 96-well plates. $TPCS_{2a}$ was added to the growth medium and the plates were incubated overnight. Cells were then resuspended in $TPCS_{2a}$-free culture medium and incubated for 3 hours. Subsequently, cells were treated for 60 minutes with increasing doses of epirubicin, gemcitabine, mitomycin C, or bleomycin followed by illumination for different periods. Cell viability was measured with a colorimetric assay after 72 hours.

Results

For the single treatments, in all 5 cell lines a dose-dependent inhibition of cell proliferation was observed. This was seen both after treatment with $TPCS_{2a}$-based photodynamic therapy (PDT), as well as after treatment with either bleomycin or one of the control chemotherapeutic agents.

After treatment with PDT (240-s illumination), bleomycin 9.0 μM, and the combination of these treatments, relative survival percentages were 89.2 ± 13.0, 70.2 ± 8.9, and 30.5 ± 6.1, respectively, in the T24 cell line. After treatment with PDT (120-s illumination), bleomycin 27 μM, and the combination of these treatments, relative survival percentages were 93.6 ± 15.7, 74.7 ± 9.6, and 30.0 ± 11.1, respectively, in the AY-27 cell line. In both cell lines, PDT combined with bleomycin showed significantly ($P < 0.001$) higher cell kill than the sum of the single treatments, suggesting a photochemical internalization effect.

Conclusions

$TPCS_{2a}$-based photochemical internalization of bleomycin showed a significant, at least, additive antiproliferative activity against human and rat urothelial carcinoma cells *in vitro*. Thus, photochemical internalization may have therapeutic potential as an intravesical strategy against bladder cancer. As the effect is heterogeneous, biomarker studies are warranted to be able to predict the effects of a photochemical internalization-based treatment.

Introduction

The initial treatment of non-muscle invasive bladder cancer (NMIBC) is transurethral resection, followed by intravesical therapy (i.e., chemotherapy or immunotherapy) [1]. However, intravesical therapy is not without toxicity, and a substantial percentage of treated patients still experiences tumour recurrences or progression to muscle-invasive bladder cancer. Therefore, improved treatment modalities are urgently needed.

Photodynamic therapy (PDT) is a potential treatment modality for NMIBC. PDT involves the administration of a photosensitizer and its subsequent activation by light of an appropriate wavelength. The result is the destruction of cells containing the photosensitizer. Clinical trials with PDT have shown promising results in the treatment of bladder cancer [2,3]. Photochemical internalization (PCI) is a new technology that can be regarded as an enhanced PDT modality. PDT and PCI share many fundamental photodynamic properties, but PCI acts as a light-directed drug-delivery system by triggered release of endocytosed macromolecules into the cytosol [4,5]. Thus, PCI can help therapeutic molecules reach their intracellular target of action, realizing their therapeutic potential, instead of being degraded by lysosomal hydrolases. The PCI effect is achieved by photosensitizing compounds specifically localizing in the membranes of endocytic vesicles, destroying these membranes by an oxidative process after illumination.

Bleomycin is used in multiple, standard cancer chemotherapy regimens, it has also been studied as intravesical treatment for NMIBC with only limited success [6,7]. The hydrophilic and relatively large chemical structure limits bleomycin's ability to penetrate membranous structures, but in many cell types bleomycin can be taken up by endocytosis. In this case, bleomycin accumulates in endocytic vesicles, where it may be degraded; or it can enter slowly into the cytosol,

where it can be degraded by bleomycin hydrolase before reaching its therapeutic target in the nucleus. However, bleomycin cytotoxicity is highly increased when the cytosol is exposed to similar amounts of bleomycin following electroporation [8]. Thus, bleomycin may become a very efficient and specific chemotherapeutic agent when it is combined with a treatment modality that activates its therapeutic potential only in the target environment. PCI of bleomycin inhibits tumour growth in different animal tumour models in a synergistic fashion [9,10].

Firstly, as bladder cancer is potentially well suited for effective treatment by PCI because it is easily accessible for both intravesical instillation and illumination, we studied the relative cell- kill effect of meso-tetraphenyl chlorin disulphonate ($TPCS_{2a}$)-based PDT [11] in 4 human bladder cancer cell lines and a rat bladder cancer cell line (anticipating future animal studies). Secondly, we studied whether $TPCS_{2a}$-based photochemical delivery of bleomycin potentiated the cytotoxicity of bleomycin on these bladder cancer cell lines. Three commonly used anti-bladder cancer intravesical chemotherapeutic agents (i.e., epirubicin, gemcitabine, and mitomycin C) were used as controls.

Materials and methods

Cell lines and culture conditions

The human urothelial carcinoma (UC) cell lines RT4, RT112, 253J, T24 [12], and rat UC cell line AY-27 (kindly provided by Dr. Ronald Moore, University of Alberta and Cross Cancer Institute, Edmonton, Alberta, Canada) were grown as a monolayer culture in RPMI-1640 medium with L-glutamine (Invitrogen, Carlsbad, CA, USA), supplemented with 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA), penicillin G (100 U/mL) and streptomycin (100 µg/mL) (Invitrogen) at 37°C in a humidified 95% air/5% carbon dioxide atmosphere. Medium was

changed twice weekly and cells were passaged using trypsin ethylenediaminetetraacetic acid (Invitrogen) when confluent.

Chemotherapeutic- and $TPCS_{2a}$-based PDT experiments

For $TPCS_{2a}$-based photodynamic treatments, cells were harvested, washed, resuspended and plated at 1.0 x 10^4 cells per well in 96-well microtiter plates (Corning Inc. Corning, NY, USA; black plate, clear bottom). $TPCS_{2a}$, (supplied by PCI Biotech AS, Oslo, Norway) was added at a final concentration of 0.2 μg/mL, and the plates were incubated overnight. Cells were then washed 3 times with $TPCS_{2a}$-free culture medium, resuspended in $TPCS_{2a}$-free culture medium and incubated for 4 hours. Cells were then illuminated for different periods (0-10 min), using a LumiSource® illumination device (PCI Biotech AS, Oslo, Norway). LumiSource® was delivered with a bank of four light tubes (4 x 18 W Osram L 18/67, Blue) emitting mainly blue light with a peak wavelength of approximately 420 nm, with an average irradiance of 13.5 mW /cm^2.

For combination therapy experiments, cells were prepared as described previously and after incubation in $TPCS_{2a}$-free culture medium for 3 hours, cells were subsequently exposed to increasing doses of epirubicin (Pfizer bv, Capelle a/d IJssel, The Netherlands) (0-48.6 μM), gemcitabine (Sun Pharmaceutical Industries Europe B.V., Hoofddorp, The Netherlands) (0-65.6 μM), mitomycin C (Kyowa Hakko Kirin Co Ltd., Tokyo, Japan) (0-65.6 μM), or bleomycin (Euro Nippon Kayaku GmbH, Frankfurt, Germany) (0-81.0 μM). All solutions were prepared on the day of use. After incubation for 1 more hour, the cells were illuminated for different periods (0-5 min) as described earlier. After illumination, cells were washed 3 times with culture medium. Cell proliferation was measured after 72 hours. Treatment doses used in these studies were selected to evaluate whether additive effects occurred and were not optimized for treatment outcome.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) assay

The effect of chemotherapy and PCI was determined by MTT assay. 72 hours after treatment, 30 µL of 5 mg/mL MTT solution (Sigma-Aldrich) prepared in phosphate-buffered saline was added to the medium. Blue dye taken up by the cells after 4 hours of incubation was dissolved in dimethyl sulfoxide (100 µL per well) and optical density at 595 nm was read on an automated microplate reader (BioRad 3550, BioRad Laboratories, Hercules, CA, USA). The bleomycin experiments were performed in quadruplicate and repeated one time by another laboratory worker; the control experiments with epirubicin, gemcitabine, and mitomycin C were performed at least once in quadruplicate.

Statistical analysis

In each combination experiment (with epirubicin, gemcitabine, mitomycin C, and bleomycin), the Wald test was used to test for statistical significance of differences in relative cell survival between the treatment groups (control, PDT alone, chemotherapy alone, and combination). $P < 0.05$ was considered statistically significant.

Results

Chemotherapeutic- and $TPCS_{2a}$-based PDT single treatment effects

PDT alone showed a clear phototoxic illumination time-dependent inhibition of cell proliferation in all 5 cell lines (figure 1). The viability of all cell lines was reduced to < 40% of control with 360-second illumination. A further increase of the light dose resulted in approximately 99% cell death at 600 seconds (data not shown).

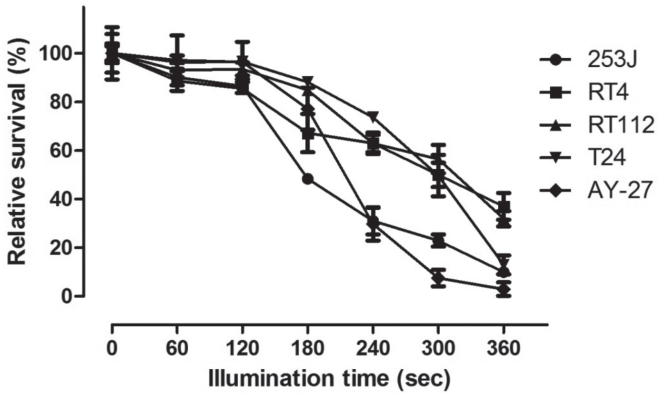


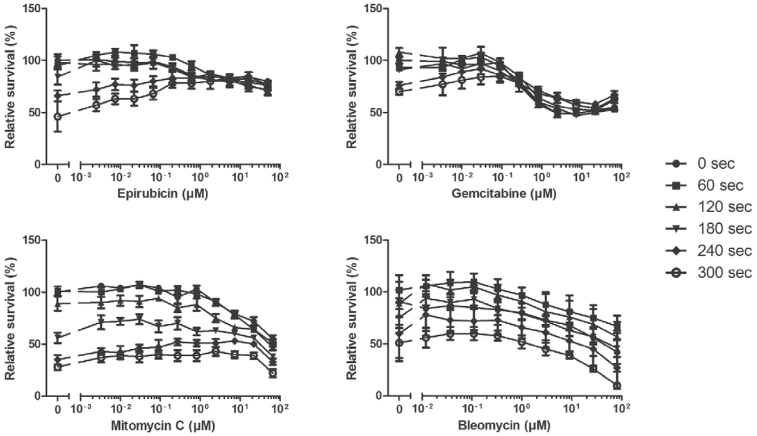
***Figure 1**: Relative survival of human 253J, RT4, RT112, and T24 and rat AY-27 urothelial carcinoma cells after $TPCS_{2a}$-based photodynamic treatment. The cells were incubated with $TPCS_{2a}$ at 0.2 µg/mL for 24 hours, washed and incubated for 4 more hours in $TPCS_{2a}$-free culture medium and subsequently illuminated. Relative cell survival was assessed 72 hours later. The percentage (mean ± SD) of viable cells from the corresponding control is shown. SD = standard deviation.*

$TPCS_{2a}$ pretreatment followed by chemotherapy treatment alone (without illumination) demonstrated a clear dose-dependent inhibition of cell proliferation in all 5 cell lines tested (figure 2).

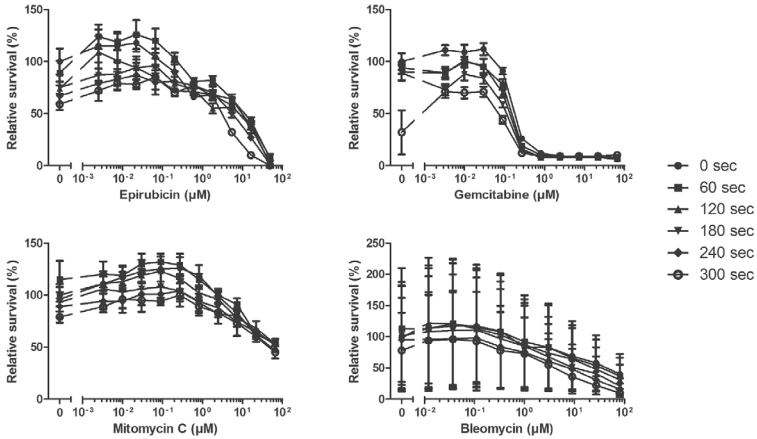
Chemotherapeutic- and $TPCS_{2a}$-based PDT combination effects

To evaluate the PCI therapy effect, chemotherapy was combined with $TPCS_{2a}$ and light treatment (figure 2). Combination of PDT and bleomycin was clearly superior to the single treatment modalities in the human T24 cell line and the rat AY-27 cell line, indicating at least an additive effect, suggesting a PCI effect. In contrast, additive treatment effects were observed for the other treatment regimens and in the other cell lines, as indicated by the parallel dose-response curves.

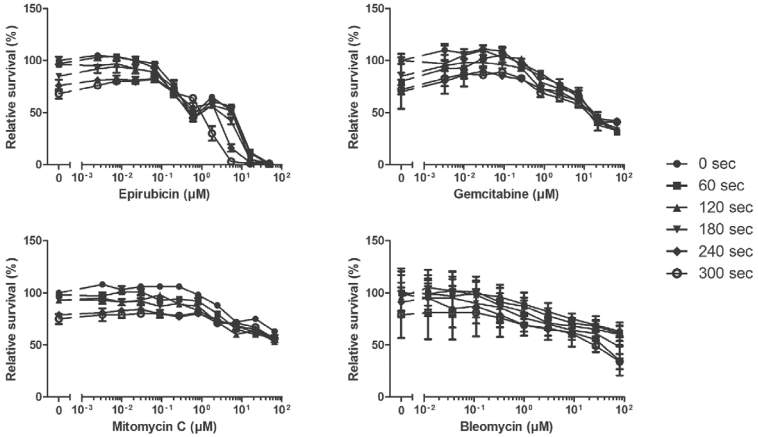
253J


Relative survival (%)
Epirubicin (μM)
Gemcitabine (μM)
Mitomycin C (μM)
Bleomycin (μM)
0 sec
60 sec
120 sec
180 sec
240 sec
300 sec

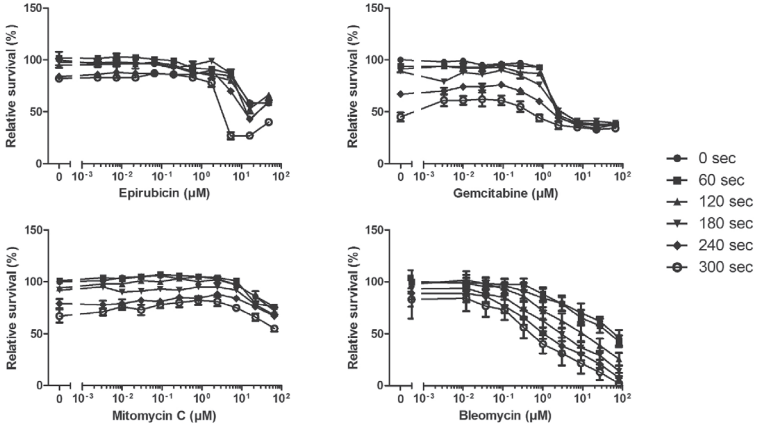
RT4


Relative survival (%)
Epirubicin (μM)
Gemcitabine (μM)
Mitomycin C (μM)
Bleomycin (μM)
0 sec
60 sec
120 sec
180 sec
240 sec
300 sec

RT112


Relative survival (%)
Epirubicin (μM)
Gemcitabine (μM)
Mitomycin C (μM)
Bleomycin (μM)
0 sec
60 sec
120 sec
180 sec
240 sec
300 sec

T24


Relative survival (%)
Epirubicin (μM)
Gemcitabine (μM)
Mitomycin C (μM)
Bleomycin (μM)
0 sec
60 sec
120 sec
180 sec
240 sec
300 sec

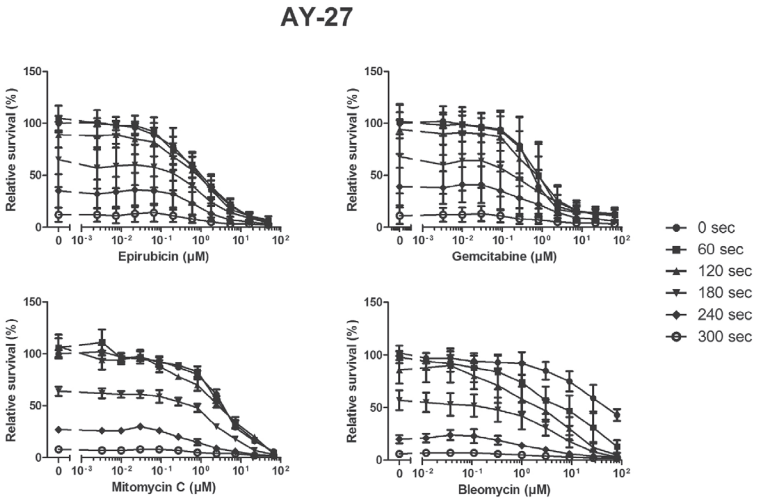


Figure 2: *Relative survival of human 253J, RT4, RT112, and T24 and rat AY-27 urothelial carcinoma cells after $TPCS_{2a}$-based photodynamic treatment in combination with epirubicin, gemcitabine, mitomycin C, or bleomycin. The cells were incubated with $TPCS_{2a}$ at 0.2 µg/mL for 24 hours, washed and incubated for 3 more hours in $TPCS_{2a}$-free culture medium and subsequently treated with an increasing dose of epirubicin, gemcitabine, mitomycin C, or bleomycin. After incubation for 1 more hour, the cells were illuminated with increasing illumination times (0-300 s). Cells were then washed and incubated again. Relative cell survival was assessed 72 hours later. The percentage (mean ± SD) of viable cells from the corresponding control is shown. The curves with 0 seconds of illumination represent the dose-response curves after treatment with chemotherapy alone. SD = standard deviation.*

The treatment effects of PDT combined with bleomycin and PDT combined with epirubicin, gemcitabine, or mitomycin C in the T24 and AY-27 cell lines are shown in figure 3. To be able to compare the 4 individual chemotherapeutic agents, we selected the chemothera-

peutic doses with a relative cell survival of > 70%: epirubicin 5.4 μM, gemcitabine 0.8 μM, mitomycin C 65.6 μM, and bleomycin 9.0 μM for the T24 cell line and epirubicin 0.2 μM, gemcitabine 0.3 μM, mitomycin C 0.8 μM, and bleomycin 27 μM for the AY-27 cell line. As shown in figure 3, PDT combined with any chemotherapeutic drug showed a significantly higher cell kill compared with PDT alone in both cell lines ($P < 0.05$). Similarly this combination was superior to chemotherapy alone ($P < 0.05$), except for epirubicin and gemcitabine treatment in the AY-27 cell line. PDT combined with bleomycin showed significantly ($P < 0.001$) higher cell kill than the sum of the single treatments in both cell lines, suggesting a PCI effect.

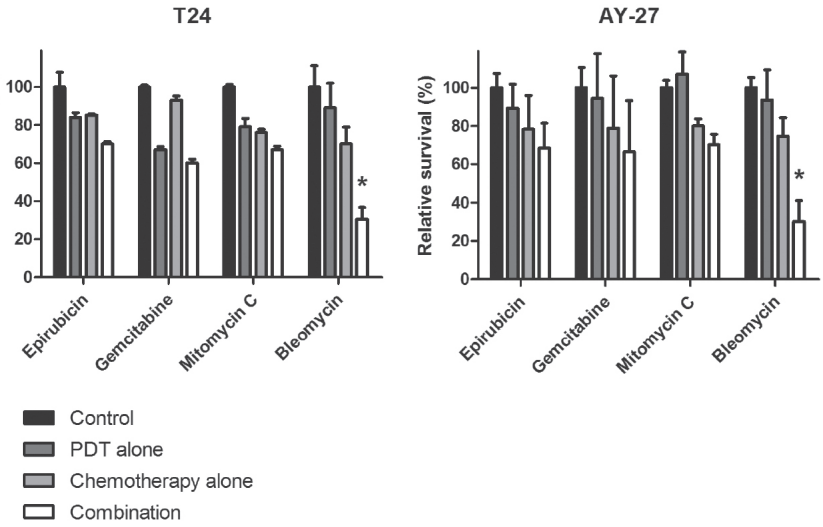


Figure 3: *$TPCS_{2a}$-based photodynamic therapy in combination with epirubicin, gemcitabine, mitomycin C, and bleomycin in the human T24 cell line and the rat AY-27 cell line. The highest chemotherapeutic doses (for chemotherapy alone) with a relative cell survival of > 70% were selected, i.e., epirubicin 5.4 μM, gemcitabine 0.8 μM, mitomycin C 65.6 μM, and bleomycin 9.0 μM in the T24 cell line and epirubicin 0.2 μM, gemcitabine 0.3 μM, mitomycin C 0.8 μM, and*

*bleomycin 27 μM in the AY-27 cell line. The chemotherapy bars represent the relative cell survival percentages after treatment with these doses. The PDT bars represent the relative cell survival percentage in the combination experiments (without chemotherapeutics) after 240-seconds illumination in the T24 cell line and after 120-seconds in the AY-27 cell line. These illumination times were selected because with these light doses the differences in relative cell survival are most clear. The combination therapy bars represent the relative cell survival percentages after the combination treatment with the selected chemotherapy and light doses. With these doses, any further added toxicity from the combination therapy can be detected and a comparison of the different combinations can be made. Each bar represents the percentage (mean + SD) of viable cells from the corresponding control. * $P < 0.001$, compared with the sum of the single treatments. SD = standard deviation.*

Discussion

In this *in situ* study, we evaluated the therapeutic potential of $TPCS_{2a}$-based photochemical delivery of bleomycin in 4 human bladder cancer cell lines and a rat bladder cancer cell line. In the T24 and AY-27 cell lines, a significant and at least additive antiproliferative effect of the combined PDT and bleomycin treatment was observed, a clear indication of the PCI effect. For all the single treatment regimens, a clear dose-dependent inhibition of cell proliferation was observed for all 5 cell lines.

Chemotherapeutics require efficient penetration into the cytosol of the tumour cells to reach their intracellular targets and exert their therapeutic activity. The prerequisite to penetrate the plasma membrane limits the exploitable chemical structure of chemotherapeutic agents to mostly small and lipophilic compounds penetrating the plasma membrane by passive diffusion. In addition, drugs (also

more hydrophilic drugs) can be taken up by means of various transporters, e.g., gemcitabine [13].

The PDT mechanism relies on the *in situ* generation of cytotoxic agents by the activation of a light-sensitive drug, resulting in cell death [14]. The PCI technology is based on the same principles as PDT, i.e., the activation of a photosensitizer by light followed by formation of reactive oxygen species. Unlike PDT, the concept of PCI is based on the use of designed photosensitizers that localize preferentially in the membranes of endocytic vesicles of the targeted cells. The most efficient PCI photosensitizers have an amphiphilic structure (e.g., $TPCS_{2a}$ [11]) with a hydrophilic part inhibiting penetration through cellular membranes. The site-specific PCI-induced drug delivery adds to the well described cytotoxic [14], vascular, and immunostimulatory effects of PDT. PCI of bleomycin has already shown to induce a synergistic inhibition of tumour growth *in vivo* [9,10]. Adigbli *et al.* [15] demonstrated an increased cytotoxic (i.e., PCI) effect of the chemotherapeutic agent mitoxantrone combined with the photosensitizer hypericin and illumination against multidrug-resistant bladder cancer cells. In general, PCI has the potential to reduce the systemic side effects of anticancer cytotoxic agents by reducing the drug dose required for a given effect and by conferring an increased level of treatment selectivity as the cytotoxic effect would be greatest in areas that are illuminated, decreasing systemic toxicity [16], in line with our observations.

A prerequisite for PCI is that the molecule of interest is accumulated in endocytic vesicles at some stage in the process. We cannot preclude that our results are the consequence of off-target effects as internalization of the chemotherapeutics was not studied here. However, bleomycin is a widely used chemotherapeutic agent for several types of cancer (e.g., testicular cancers, malignant lymphomas and squamous cell carcinomas of the cervix, and head and neck cancers), with endocytosis being an important uptake mechanism [8], proba-

bly because of its hydrophilic and relatively large chemical structure (molecular weight: 1,415.6 Da). Thus, not unexpectedly we observed the most pronounced PCI effects with bleomycin. The lower PCI effects seen for the other chemotherapeutics are most likely the consequence of their lower molecular weight and higher hydrophobicity, or their cellular uptake is dependent on the activity of transport proteins, promoting easier cytoplasmic accumulation.

Only in the T24 and AY-27 cell lines, the treatment effects of PDT combined with bleomycin were significantly higher than the sum of the single treatments. The heterogeneous response of the bladder cancer cell lines is probably a reflection of the heterogeneity of the disease. It is possible that the endocytosis and transport mechanisms of bleomycin or $TPCS_{2a}$ or both differ between the various cell lines. It has been well established that the cytotoxic effect of bleomycin varies widely between different tumours as well as between different organs [17]. This might be due to differences in the cells' DNA-repair capacity, bleomycin hydrolase activity, cellular uptake mechanisms, and possibly the rate of bleomycin efflux. In our experiments, $TPCS_{2a}$ represents an additional element. It is possible that different cell types use different endocytosis pathways leading to intracellular $TPCS_{2a}$ accumulation resulting in cell death without endosomal release. Another possible factor for the heterogeneous antiproliferative effects are the cellular resistance mechanisms to bleomycin. A better understanding of tumour biology and pathways critical for tumour genesis may provide personalized treatment opportunities for patients with urothelial cancer.

Conclusion

$TPCS_{2a}$-based PCI of bleomycin showed a significant, at least, additive antiproliferative activity against human and rat UC cells *in vitro*. Therefore, PCI may have therapeutic potential as an intraves-

ical strategy against NMIBC, provided patient stratification can be achieved based on a predictive biomarker (panel). Further studies are needed to explore whether such predictive biomarkers can be defined.

References

1. Babjuk M, Oosterlinck W, Sylvester R *et al.*; European Association of Urology (EAU).
   EAU guidelines on non-muscle-invasive urothelial carcinoma of the bladder, the 2011 update. Eur Urol 2011;59:997-1008
2. Yavari N, Andersson-Engels S, Segersten U *et al.*
   An overview on preclinical and clinical experiences with photodynamic therapy for bladder cancer. Can J Urol 2011;18:5778-5786
3. Bozzini G, Colin P, Betrouni N *et al.*
   Photodynamic therapy in urology: What can we do now and where are we heading? Photodiagnosis Photodyn Ther 2012;9:261-273
4. Berg K, Selbo PK, Prasmickaite L *et al.*
   Photochemical internalization: a novel technology for delivery of macromolecules into cytosol. Cancer Res 1999;59:1180-1183
5. Norum OJ, Selbo PK, Weyergang A *et al.*
   Photochemical internalization (PCI) in cancer therapy: from bench towards bedside medicine. J Photochem Photobiol B 2009;96:83-92
6. Bracken RB, Johnson DE, Rodriquez L *et al.*
   Treatment of multiple superficial tumors of bladder with intravesical bleomycin. Urology 1977;9:161-163
7. Tripi M, Pavone C, Ammatuna P *et al.*
   Intravesical bleomycin in bladder tumour prophylaxis. Int Urol Nephrol 1986;18:411-416
8. Pron G, Mahrour N, Orlowski S *et al.*
   Internalisation of the bleomycin molecules responsible for bleomycin toxicity: a receptor-mediated endocytosis mechanism. Biochem Pharmacol 1999;57:45-56
9. Berg K, Dietze A, Kaalhus O *et al.*
   Site-specific drug delivery by photochemical internalization enhances the antitumor effect of bleomycin. Clin Cancer Res 2005;11:8476-8485

10. Norum OJ, Bruland ØS, Gorunova L *et al.*
Photochemical internalization of bleomycin before external-beam radiotherapy improves locoregional control in a human sarcoma model. Int J Radiat Oncol Biol Phys 2009;75:878-885
11. Berg K, Nordstrand S, Selbo PK *et al.*
Disulfonated tetraphenyl chlorin ($TPCS_{2a}$), a novel photosensitizer developed for clinical utilization of photochemical internalization. Photochem Photobiol Sci 2011;10:1637-1651
12. Masters JRW, Hepburn PJ, Walker L *et al.*
Tissue culture model of transitional cell carcinoma: characterization of twenty-two human urothelial cell lines. Cancer Res 1986;46:3630-3636
13. Candelaria M, De la Cruz-Hernández E, Pérez-Cárdenas E *et al.*
Pharmacogenetics and pharmacoepigenetics of gemcitabine. Med Oncol 2010;27:1133-1143
14. Selbo PK, Kaalhus O, Sivam G *et al.*
5-Aminolevulinic acid-based photochemical internalization of the immunotoxin MOC31-gelonin generates synergistic cytotoxic effects *in vitro*. Photochemistry and Photobiology 2001;74:303-310
15. Adigbli DK, Wilson DGG, Farooqui N *et al.*
Photochemical internalisation of chemotherapy potentiates killing of multidrug resistant breast and bladder cancer cells. British journal of cancer 2007;97:502-512
16. Høgset A, Prasmickaite L, Selbo PK *et al.*
Photochemical internalisation in drug and gene delivery. Adv Drug Deliv Rev 2004;56:95-115
17. Chen J and Stubbe J.
Bleomycins: towards better therapeutics. Nat Rev Cancer 2005;5:102-112

Chapter 3

Experimental rat bladder urothelial cell carcinoma models

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World J Urol. 2009;27(3):313-317

Abstract

Bladder cancer is a major public health problem. Currently available therapeutic options seem to be unable to prevent bladder cancer recurrence and progression. To enable preclinical testing of new intravesical therapeutic agents, a suitable bladder tumour model that resembles human disease is highly desirable. The aim of this topic paper was to discuss the problems associated with current *in vivo* animal bladder tumour models, focusing on the orthotopic syngeneic rat bladder tumour model. In the second part of the paper the development of a potential new orthotopic rat bladder tumour model is described.

Introduction

Bladder cancer is one of the most common malignancies with approximately 357,000 new cases (273,858 males and 82,699 females) worldwide in 2002 [1]. More than 90% of the bladder cancers are urothelial cell carcinomas (UCC) [2] and the majority (75-85%) presents initially as non-muscle invasive bladder cancer (NMIBC) [3]. Of these patients, about 70% presents with papillary lesions that are non-invasive and limited to the mucosa (Ta), 20% with lesions that invade the subepithelial connective tissue (T1) and 10% with carcinoma *in situ* (CIS). Transurethral resection of the bladder tumour (TURBT), followed by adjuvant intravesical instillations with chemotherapy and/or immunotherapy are considered standard treatment for NMIBC. In general, the probabilities of recurrence and progression in NMIBC at 5 years range from 31 to 78% and from less than 1% to 45% depending on grade and stage, respectively [4]. These rates illustrate the modest success of currently available treatments and underline the need for improved adjuvant treatment.

Although *in vitro* models are useful for initial development and evaluation of therapeutic agents and modalities, adequate animal models are still essential in the preclinical development of new effective and safe therapies for many human malignancies. It allows the investigation of aspects that cannot be studied under clinical conditions such as pharmacokinetics and toxicity. For bladder cancer in particular, much effort has been devoted to the development of an appropriate tumour model for the evaluation of new chemotherapeutic or immunotherapeutic agents, drug regiments, or other anti-tumour modalities.

The ideal animal bladder tumour model that resembles human disease both histologically and in behaviour should include the following characteristics [5]:

1. The tumour should grow intravesically (orthotopically), such that

the tumour can be directly exposed to intravesical anti-tumour drugs in its natural environment.
2. The tumour should be of pure UCC origin, with different stages of disease progression (CIS, papillary and invasive diseases) and, as for the human disease, the majority of the tumours should be non-muscle invasive, but not progressive.
3. The animal host should be immunocompetent and reasonably large, so it can be treated by various anti-tumour modalities such as immunotherapy with bacillus Calmette–Guérin (BCG), chemotherapy, and whole bladder photodynamic therapy (PDT).
4. The tumour should be technically easy to develop within a reasonable time period, and highly reproducible with respect to its natural history.

In this topic paper, we discuss existing *in vivo* rat bladder tumour models with their specific shortcomings. In the second part of the paper, we describe the development of a potential new orthotopic rat bladder tumour model.

Existing in vivo models

Rats and mice are the most common species used for *in vivo* UCC models. Rodents have a lower urinary tract comparable to humans and neoplasms in the bladder are morphologically very alike [6]. Bladder tumours in rodents can be established subcutaneously (heterotopically) or in the bladder (orthotopically) either by transplantation of tumour cells or by chemical induction.

Although murine orthotopic models are available [e.g. 7,8,9], these models are limited by the size of the animal, with a small urethral caliber, thin bladder wall and small bladder capacity as a consequence. Catheterization of mice to introduce tumour cells, intravesical therapeutics or for example an ultrathin cystoscope is more

difficult than in rats. A rat bladder is approximately 10 times larger than the bladder of a mouse and the better developed muscular layer allows better histological assessment of depth of invasion and decreases the risk of perforation during bladder catheterization [10]. Therefore, in important aspects the rat model offers significant advantages over the murine model and in this topic paper only the rat model is further discussed.

Subcutaneous models

In rodent heterotopic UCC models, the tumour is usually located in the flank or hind leg of the animal. To establish a syngeneic subcutaneous model for immunological studies, rodent UCC tumour fragments or cells are inoculated through a small incision into the immunocompetent host of the same strain from which the tumour was originally derived [11,12]. For a xenograft model human UCC fragments or cells are used. Subcutaneous tumours can be evaluated non-invasively by palpation or with the help of imaging techniques. Treatment can be administered by local injection or systemically.

Subcutaneous bladder tumour models have been widely used because of the ease of assessing tumour growth kinetics and because the orthotopic model is technically more difficult (see below) [13,14]. However, the microenvironment at the implantation site of the host organ can influence the natural history of tumour growth and the efficacy of anti-proliferative agents. In one study, for example [15], human colon carcinoma cells were implanted into different anatomical locations (subcutaneous and cecum) of nude mice. Tumour bearing mice were treated with doxorubicin and subsequently evaluated for responses. The heterotopic subcutaneous tumours showed an 80% inhibition of growth, compared with 40% inhibition of the orthotopic intracecal tumours. The environment of the orthotopic model resembles that of naturally occurring tumours, so experimental results in this model would be expected to have more relevance than results in the subcutaneous model. In addition, heterotopic

grafts are unsuited to evaluate intravesical therapies and orthotopic models are thus to be preferred.

Orthotopic models: tumour development

To test potential new drugs against NMIBC in a preclinical setting, a clinically relevant rodent orthotopic bladder tumour model is highly desirable. Currently, there are three different kinds of orthotopic rat bladder tumour models: chemically induced bladder cancer models [16], the xenograft model (transplantation of human UCC into immunodeficient nude rats) [10] and the syngeneic tumour model (transplantation of carcinogen-induced bladder cancer in syngeneic immunocompetent rats) [5].

Given the importance of chemicals in the carcinogenesis of bladder cancer, organo-specific bladder carcinogens were discovered for rodents. These chemicals provided readily available reproducible models necessary for detailed studies of the pathogenesis of bladder cancer [17]. Three chemicals have been reported as being particularly effective in causing bladder tumours under the appropriate conditions: FANFT (*N*-[4-(5-nitro-2-furyl)-2-thiazolyl]formamide), OH-BBN (*N*-butyl-*N*-(4-hydroxybutyl)-nitrosamine) and MNU (*N*-methyl-*N*-nitrosurea) [18,19]. However, the use of chemicals to induce tumours in the rat bladder is time-consuming (several months) and there is a high amount of squamous differentiation. Furthermore, reproducibility is hampered by the variation in time to tumour development. Therefore, these models are less practical in therapeutic efficacy studies of novel anti-proliferative compounds.

Bladder tumour transplantation is a much faster approach to create a therapeutic *in vivo* orthotopic UCC model. Orthotopic xenografts in immunodeficient nude rats are useful in order to better approximate human tumour cell behaviour *in situ*, but are unsuited to test intravesical immunotherapy with for example BCG and are not frequently used.

Xiao *et al.* [5] described a syngeneic orthotopic rat model resembling human UCC, with reproducible tumour growth. The rat bladder UCC cell line AY-27 was established as a primary bladder tumour in Fischer F344 rats by feeding FANFT. This rat bladder UCC cell line was transplanted orthotopically into female Fischer F344 rats. AY-27 UCC cells were grown in monolayer cell culture and instilled intravesically as single cell suspensions into bladders that had been preconditioned with mild acid washing. In 82 animals sacrificed at 16 days, 80/82 (97%) developed UCC, and in 52/80 (65%) the tumour was non-muscle invasive (CIS, T1, but no Ta). Histological examination of the tumour specimens confirmed the presence of grade II-III UCC. Immunocytochemistry confirmed that the tumour model maintained the features of UCC.

A potential demerit in this frequently used model is the timing of intravesical therapy after tumour cell inoculation: in some rats tumours had not formed yet, while other rats already developed muscle invasive tumours. Therefore, Hendricksen *et al.* [20] assessed the growth of UCC in this model over time, aiming to have a maximum number of rats with NMIBC at a particular time and thus optimize the future starting point of experimental intravesical treatment. It was shown that in this model tumours were formed at 3-5 days and remained non-muscle invasive up to 5 days. From 6 days, tumours progressed to muscle-invasive disease in 40% of the rats. The observed increase in tumour aggressiveness might be explained by more passages *in vitro*.

Another disadvantage of transplantable models is that tumours prove to be invasive carcinomas (≥ T1) from the start, without any Ta tumours (which is the most common stage in human NMIBC). This is related to the bladder preconditioning with urothelial abrasion rather than to the aggressiveness of the AY-27 cell line. It is quite possible that the basal membrane is disrupted, which causes rapid invasion of tumour cells into the underlying layers of the bladder.

However, tumour cells instilled into normal (non-conditioned) bladders did not result in tumour establishment [5]. Different methods of tumour establishment in orthotopic models have been described, ranging from intravesical instillation of tumour cell suspension after chemical urothelial denudation with MNU or the combination of HCl and KOH leading to multifocal lesions, to mechanical urothelial lesioning such as cauterization [21]. Intraparietal injection of tumour cells resulted in intramuscular tumours only [22,23]. Horiguchi *et al.* [24] however, reported also promising success rates without bladder preconditioning. Thus, the model could possibly be improved by controlling the urothelial abrasion: the lesion should be only superficial, affecting only one or two of the three cell layers of the rat bladder urothelium. On the other hand, if one assumes that tumour cell implantation on the urothelium injured during TURBT is a major cause of bladder cancer recurrence in humans, bladder preconditioning would resemble the clinical situation in case the model is used to test immediate postoperative intravesical treatments.

Orthotopic models: tumour evaluation

For proper determination of treatment response, confirmation of successful orthotopic tumour implantation before starting treatment is desirable. Otherwise one has to rely on former experiments and conjecture stage and tumour take. Since there is no accurate documentation of the presence or extent of tumour before therapy in this case, the validity of any conclusion on the efficacy of the experimental agents is difficult and large experimental groups are needed to reach enough power. If the autopsy specimen after treatment contained no tumour one could not conclude whether complete tumour regression was caused by therapy or whether tumour implantation had failed. Therefore, a placebo group should always be added in treatment efficacy experiments in case successful tumour implantation before starting treatment could not be assessed. In any case, there is the disadvantage of consuming more animals, either due to larger treatment groups or to adding a placebo group.

Precise quantification of bladder tumour lesions is also needed to assess responses to intravesical instillation of therapeutic agents. Accurate non-invasive *in vivo* assessment of established tumours in the orthotopic model is difficult, particularly for early stage non-muscle invasive tumours. Magnetic resonance imaging (MRI) has been reported for this purpose [25, 26]. However, until now it could not offer an accurate diagnosis of small early lesions (< 1 mm in diameter) due to its spatial resolution. Furthermore, it is relatively complicated and has high costs. Intravesical ultrasonography was also reported [27], and provided a positive-predictive ratio of tumour stage up to 85%, however, without information on tumour location and appearance. Bioluminescence imaging has the potential to become a valuable tool for the early detection of tumour growth, although bioluminescence was only first detectable on day 4 after tumour cell implantation in recently published studies [28,29]. Ultrathin cystoscopy [30] is a reliable method in diagnosing tumour growth in an orthotopic rat bladder tumour model, with a specificity and sensitivity of > 90% [20]. Unfortunately, in our own experience, cystoscopy is suboptimal in the follow-up of tumours after intravesical treatment in this model. Most probably the rather short interval between intravesical instillation and cystoscopy, necessary because of the fast tumour development, hampers reliable cystoscopy. This lack of a reliable method to follow-up tumour development makes it difficult to study treatment outcomes without sacrificing the animals, because the histological examination of the bladder appears to remain the golden standard for the determination of tumour growth.

Potential new orthotopic ACI rat bladder tumour (RBT323) model

In the search for the ideal animal bladder cancer model, we are currently developing a new orthotopic ACI rat bladder tumour (RBT323) model. The RBT323 tumour is a non-carcinogen-induced tumour and

arose spontaneously in an ACI rat. In the past, this RBT323 tumour was used as a subcutaneous rat bladder tumour model in our laboratory [31,32]. Histologically, the RBT323 tumour is a grade 2-3 pure urothelial cell tumour with no squamous cells and the RBT323 cell line closely resembles human non-muscle invasive UCC both phenotypically and cytogenetically [11].

The rather short treatment window of the frequently used syngeneic orthotopic AY-27/Fischer F344 rat bladder tumour model hampers its use in the evaluation of new treatments for NMIBC [5]. Moreover, it was recently shown that carcinogen-induced rodent bladder tumours have gene expression profiles more similar to those of human invasive than non-invasive tumours and thus would be a better experimental model for the former [33]. For these reasons, we anticipated an orthotopic RBT323 tumour in the ACI rat to be an appropriate syngeneic model to study the anti-tumour effects of possible new treatments.

To accomplish this, a RBT323 rat bladder UCC cell line was established and transplanted orthotopically into syngeneic female ACI/SeqHSD rats (Harlan Spraque Inc., Indianapolis, Ind, USA). RBT323 UCC cells were grown in monolayer cell culture and instilled into preconditioned (mild acid washing) rat bladders as single cell suspensions. Tumour growth was assessed by ultrathin cystoscopy (0,5 mm diameter) and 24 days after instillation of tumour cells all rats were sacrificed and subjected to necropsy. Cystectomy was performed for histopathological examination. Overall in three out of the four rats tumour was established. All tumours were non-muscle invasive (T1a-T1c) except one T2a tumour (2002 TNM staging system). No metastases were found. Unfortunately, all tumours were covered with normal urothelium (figure 1). Still, initial results of this new orthotopic rat bladder tumour model are promising, because after 24 days most of the tumours seem to be non-muscle invasive with a reasonably long time window for the administration of study drugs,

as a consequence. Further studies are now being performed to look for the reproducibility and longitudinal behavior of the model. Additionally, a study is performed without bladder preconditioning, but with optimized other transplantation conditions, trying to establish Ta tumours.

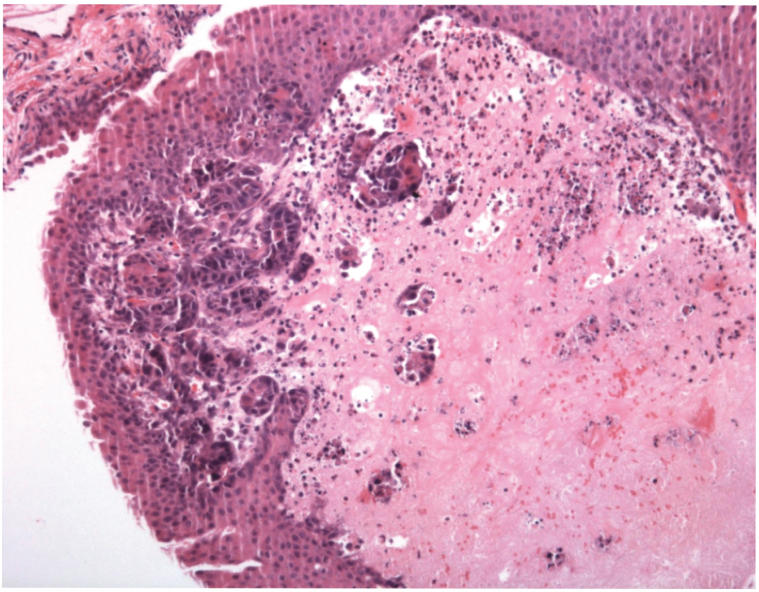

Figure 1: *RBT323 rat UCC T1a tumour, with overlying normal urothelium.*

Conclusion

To enable efficacy testing of new potential chemotherapeutic or immunotherapeutic agents, an orthotopic syngeneic non-muscle invasive UCC rat bladder tumour model would be ideal. However, especially tumour cell implantation methods and diagnostic procedures for the early detection of tumour growth, and follow-up monitoring still need to be refined.

References

1. Curado MP, Edwards B, Shin HR *et al.*
   Cancer incidence in five continents, Vol. IX. IARC Scientific Publications No.160. IARC, Lyon, 2007
2. Fleshner NE, Herr HW, Stewart AK *et al.*
   The national cancer data base report on bladder carcinoma. Cancer 1996;78:1505-1513
3. Babjuk M, Oosterlinck W, Sylvester R *et al.*
   Guidelines on TaT1 (non-muscle invasive) bladder cancer. Eur Urol 2008;54:303-314
4. Sylvester RJ, van der Meijden AP, Oosterlinck W *et al.*
   Predicting recurrence and progression in individual patients with stage Ta T1 bladder cancer using EORTC risk tables: a combined analysis of 2596 patients from seven EORTC trials. Eur Urol 2006;49:466-477
5. Xiao Z, McCallum TJ, Brown KM *et al.*
   Characterization of a novel transplantable orthotopic rat bladder transitional cell tumour model. Br J Cancer 1999;81:638-646
6. Oyasu R.
   Epithelial tumours of the lower urinary tract in humans and rodents. Food Chem Toxicol 1995;33:747-755
7. Günther JH, Jurczok A, Wulf T *et al.*
   Optimizing syngeneic orthotopic murine bladder cancer (MB49). Cancer Res 1999;59:2834-2837
8. Watanabe T, Shinohara N, Sazawa A *et al.*
   An improved intravesical model using human bladder cancer cell lines to optimize gene and other therapies. Cancer Gene Ther 2000;7:1575-1580
9. Chong L, Ruping Y, Jiancheng B *et al.*
   Characterization of a novel transplantable orthotopic murine xenograft model of a human bladder transitional cell tumor (BIU-87). Cancer Biol Ther 2006;5:394-398
10. Oshinsky GS, Chen Y, Jarrett T *et al.*

A model of bladder tumor xenografts in the nude rat. J Urol 1995;154:1925-1929
11. Van Moorselaar RJA, Ichikawa T, Schaafsma HE *et al.*
The rat bladder tumor model system RBT resembles phenotypically and cytogenetically human superficial transitional cell carcinoma. Urol Res 1993;21:413-421
12. Moltedo B, Faunes F, Haussmann D *et al.*
Immunotherapeutic effect of Concholepas hemocyanin in the murine bladder cancer model: evidence for conserved antitumor properties among hemocyanins. J Urol 2006;176:2690-2695
13. Talmadge JE, Singh RK, Fidler IJ *et al.*
Murine models to evaluate novel and conventional therapeutic strategies for cancer. Am J Pathol 2007;170:793-804
14. Bibby MC.
Orthotopic models of cancer for preclinical drug evaluation: advantages and disadvantages. Eur J Cancer 2004;40:852-857
15. Wilmanns C, Fan D, O'Brian C *et al.*
Modulation of doxorubicin sensitivity and level of P-glycoprotein expression in human colon carcinoma cells by ectopic and orthotopic environments in nude mice. Int J Oncol 1993;3:413-422
16. Grubbs CJ, Lubet RA, Koki AT *et al.*
Celecoxib inhibits *N*-Butyl-*N*-(4-hydroxybutyl)-nitrosamine-induced urinary bladder cancers in male B6D2F1 mice and female Fischer-344 rats. Cancer Res 2000;60:5599-5602
17. Cohen SM.
Cell proliferation and carcinogenesis. Drug Metab Rev 1998;30:339-357
18. Schalken JA, Van Moorselaar RJA, Bringuier PP *et al.*
Critical review of the models to study the biologic progression of bladder cancer. Semin Surg Oncol 1992;8:274-278
19. Oliveira PA, Colaço A, De la Cruz LF *et al.*
Experimental bladder carcinogenesis-rodent models. Exp Oncol 2006;28:2-11
20. Hendricksen K, Molkenboer-Kuenen J, Oosterwijk E *et al.*

Evaluation of an orthotopic rat bladder urothelial cell carcinoma model by cystoscopy. BJU Int 2008;101:889-893
21. Bisson J, Parache RM, Droulle P *et al.*
A new method of implanting orthotopic rat bladder tumor for experimental therapies. Int J Cancer 2002;102:280-285
22. Iinuma S, Bachor R, Flotte T *et al.*
Biodistribution and phototoxicity of 5-aminolevulinic acid-induced PpIX in an orthotopic rat bladder tumor model. J Urol 1995;153:802-806
23. Bindels EMJ, Vermey M, De Both NJ *et al.*
Influence of the microenvironment on invasiveness of human bladder carcinoma cell lines. Virchows Arch 2001;439:552-559
24. Horiguchi Y, Kikuchi E, Ozu C *et al.*
Establishment of orthotopic mouse superficial bladder tumor model for studies on intravesical treatments. Hum Cell 2008; 21:57-63
25. Chin J, Kadhim S, Garcia B *et al.*
Magnetic resonance imaging for detecting and treatment monitoring of orthotopic murine bladder tumor implants. J Urol 1991;145:1297-1301
26. Kikuchi E, Xu S, Ohori M *et al.*
Detection and quantitative analysis of early stage orthotopic murine bladder tumor using *in vivo* magnetic resonance imaging. J Urol 2003;170:1375-1378
27. Satoh H, Morimoto Y, Arai T *et al.*
Intravesical ultrasonography for tumor staging in an orthotopically implanted rat model of bladder cancer. J Urol 2007;177:1169-1173
28. Hadaschik BA, Black PC, Sea JC *et al.*
A validated mouse model for orthotopic bladder cancer using transurethral tumor inoculation and bioluminescence imaging. BJU Int 2007;100:1377-1384
29. Jurczok A, Fornara P and Söling A.
Bioluminescence imaging to monitor bladder cancer cell adhe-

sion *in vivo*: a new approach to optimize a syngeneic orthotopic, murine bladder cancer model. BJU Int 2007;101:120-124
30. Asanuma H, Arai T, Seguchi K *et al*.
Successful diagnosis of orthotopic rat superficial bladder tumor model by ultrathin cystoscopy. J Urol 2003;169:718-720
31. Van Moorselaar RJA, Hendriks BT, Borm G *et al*.
Inhibition of rat bladder tumor (RBT323) growth by tumor necrosis factor alpha and interferon-gamma in vivo. J Urol 1992;148:458-462
32. Cornel EB, Van Moorselaar RJA, Van Stratum P *et al*.
Antitumor effects of Bacillus Calmette-Guerin in a syngeneic rat bladder tumor model. J Urol 1993;149:179-182
33. Williams PD, Lee JK and Theodorescu D.
Molecular credentialing of rodent bladder carcinogenesis models. Neoplasia 2008;10:838-846

Chapter 4

The orthotopic Fischer/AY-27 rat bladder urothelial cell carcinoma model to test the efficacy of different apaziquone formulations

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Abstract

Objective

Apaziquone used intravesically showed significant activity in phase I and II marker lesion studies in non-muscle invasive bladder cancer. The objective of this study was to assess anti-tumour activity and safety of 3 different formulations of intravesical apaziquone in an orthotopic rat bladder cancer model.

Animals, materials and methods

Female Fischer F344 rats were instilled with 1.5×10^6 AY-27 urothelial cell carcinoma cells and divided in 3 treatment groups (n = 10) and 1 placebo group (n = 6). Intravesical treatment was administered for 1 hour on days 2 and 5. Rats were treated with apaziquone in the formulation used in phase I/II clinical trials (group 1); apaziquone with an altered buffering capacity being used in phase III clinical trials (group 2), and apaziquone as in group 2, but without propylene glycol in the diluent (group 3). On days 5 and 14, the bladder wall was inspected by cystoscopy and evaluated for macroscopic tumour growth. After sacrificing the rats (day 14), cystectomy was performed and the bladders were investigated.

Results

There were no signs of any toxicity due to the study drug. On histopathologic examination of the bladders 0, 1, and 2 tumours per group were found in group 1, 2, and 3, respectively. In the placebo-treated group, 60% of animals developed tumour, which is comparable to untreated animals.

Conclusions

Apaziquone showed an excellent anti-tumour activity. The effectiveness of apaziquone in this orthotopic rat bladder tumour model corroborates the clinical observations and implies the validity of this model.

Introduction

Bladder cancer is the fourth most common malignancy in men, with an estimated 68,810 new cases and 14,100 deaths in the USA in 2008 [1]. The majority of patients (around 70%) initially present with non-muscle invasive bladder cancer (NMIBC) [2]. Transurethral resection of the bladder tumour (TURBT), possibly followed by adjuvant intravesical instillations with chemotherapy and/or immunotherapy are standard treatment for patients with NMIBC. The probabilities of recurrence and progression in NMIBC at 5 years after standard treatment range from 31% to 78% and from less than 1% to 45% depending on grade and stage, respectively [3]. These rates illustrate the modest success of currently available treatments and underline the need for improved adjuvant treatment.

Apaziquone, 3-hydroxymethyl-5-aziridinyl-1-methyl-2-(1*H*-indole-4,7-dione)-prop-β-en-α-ol, is a synthetic bioreductive alkylating indoloquinone. As a pro-drug it generates cytotoxic species (semiquinone/hydroquinone) after enzymatic activation by the enzyme DT-diaphorase. Apaziquone has good anti-proliferative activity *in vitro* [4] but has only a modest anti-tumour activity in *in vivo* animal models and also in human clinical trials when the drug was given intravenously [5-7]. The lack of activity was mainly attributed to the rapid clearance of the drug (plasma half-life < 10 minutes) and poor drug delivery to tumour cells. When apaziquone was administered by intravesical instillation, it showed impressive activity in phase I and phase II marker lesion studies in NMIBC [8,9]. Although the activity of apaziquone has been demonstrated in marker lesion studies, to date apaziquone has not been tested in orthotopic bladder cancer models.

Apaziquone stability in urine is best at pH 8.5, and the drug becomes progressively less stable as pH becomes more acidic [10,11], which might influence anti-tumour efficacy. Propylene glycol in the

diluent improves the solubility of apaziquone, but the presence of propylene glycol might also influence anti-tumour efficacy as it significantly impairs the penetration of apaziquone [12]. The objective of the current study was to assess efficacy and safety of 3 different formulations of intravesically installed apaziquone with slightly different buffer compositions, to improve drug stability, and propylene glycol content in the diluent, to improve solubility, in an orthotopic rat bladder cancer model [13].

Animals, materials, and methods

Animals

Animal procedures were performed according to the protocol approved by the Institutional Animal Care and Use Committee (IACUC, Radboud University Nijmegen Medical Centre, The Netherlands) and in compliance with national and European regulations. A total of 36 female Fischer F344 rats (Charles River, L'Arbresle Cedex, France) weighing 154-189 g were housed in individual cages (Techniplast, Milan, Italy) with goldflakes bedding (SPPS, Frasne, France) and environmental enrichment, in a temperature controlled environment with a 12-hour light/dark cycle with free access to standard chow and water.

Tumour cell line

The AY-27 urothelial cell carcinoma cell line was established as a primary bladder tumour in FANFT (*N*-[4-(5-nitro-2-furyl)-2-thiazolyl] formamide) fed Fischer F344 rats. Cells were grown as a monolayer culture in RPMI-1640 medium with L-glutamine, supplemented with 10% fetal bovine serum, 100 U/mL penicillin G, and 100 µg/mL streptomycin at 37°C in a humidified 95% air/5% carbon dioxide atmosphere.

Tumour cell implantation

The instillation procedure was essentially performed as described by Xiao *et al*. [14], resulting in greater than 80% bladder tumour establishment [13]. Experimental procedures were performed under inhalation anaesthesia: isoflurane 2%-5% (induction) followed by isoflurane 2%, nitric oxide 0.5 L/min, and oxygen 1 L/min. Before each catheterization, enrofloxacin (5-10 mg/kg) was injected subcutaneously for antibacterial prophylaxis. The rat bladder was catheterized via the urethra with a 16-gauge (1.4 mm) plastic intravenous cannula (BD Biosystems, Erembodegem-Aalst, Belgium) and drained. The bladder was preconditioned with a 15 s instillation of 0.4 mL 0.1 M hydrochloride (HCl) and neutralized by adding 0.4 mL 0.1 M potassium hydroxide (KOH) for 15 s. The bladder was then drained and flushed 3 times with 0.8 mL 0.01 M phosphate-buffered saline (PBS). Immediately after bladder conditioning, freshly harvested AY-27 cells (passage 38) (1.5×10^6 cells in 0.5 mL medium, time between cell harvest and bladder inoculation less than 2 hours) were instilled via the catheter and left indwelling for 60 minutes. Leakage of the cell suspension around the catheter was carefully monitored. Every 15 minutes, rats were rotated 90° to facilitate full bladder wall exposure. The catheter was removed after 60 minutes and the rats were allowed to void spontaneously. The well-being of the animals was monitored on a daily basis, with special emphasis on haematuria and weight loss.

Test agents

Apaziquone and solvents were supplied by Spectrum Pharmaceuticals, Inc. (Irvine, CA, USA). All lyophilized powders and solvents for reconstitution were maintained in the dark at 4°C and prepared fresh prior to each instillation.

Experimental groups

Rats were randomly assigned into experimental groups. Assuming a cure rate of 67% (complete response rate in clinical phase II marker lesion study [8]), a tumour yield after tumour cell implantation

> 80% (model evaluation [13]), $\alpha = 0.05$ and a power of 80%, sample size of treatment groups was estimated 10 (possible drop outs taken into account). Rats were intravesically treated 2 and 5 days after instillation of the tumour cells. Treatment consisted of (1) 0.05 mg apaziquone (formulation used in phase I/II clinical trials)/0.5 mL solvent (group 1, $n = 10$), (2) 0.05 mg apaziquone (different buffering capacity, used in phase III clinical trials)/0.5 mL solvent (group 2, $n = 10$), (3) 0.05 mg apaziquone as in group 2, but without propylene glycol in the diluent/0.5 mL 5 mg/mL sodium bicarbonate (group 3, $n = 10$), and (4) 0.05 mg placebo/0.5 mL 0.9% saline (group 4, $n = 6$). The placebo used was identical to the placebo used in the apaziquone clinical trials, containing the red dye FD and C Red no. 40, to match the color of the reconstituted apaziquone solution. The solvent used in groups 1 and 2 contained 30% vol/vol propylene glycol. One rat in the placebo group died during anaesthesia on day 0 and was excluded from the study. One rat in group 2 also died during anaesthesia although on day 5, and one rat in group 3 was sacrificed on day 9 because the humane endpoint was reached. These two rats died before reaching the end of the experiment, but after they received both treatments, and were therefore not excluded from the study.

Tumour assessment

Macroscopic tumour growth was assessed by cystoscopy on day 5 (before bladder instillation) and day 14 (before necropsy). A fiber optic needle arthroscope (Karl Storz, Tuttlingen, Germany) with 1.0 mm diameter, 6.5 cm length, and miniature-straight forward 0° telescope was used. The catheter functioned as sheath with an airtight rubber plug connected to it. The cystoscope was inserted and the bladder distended with 0.8 mL of air. The bladder surface was inspected systematically, and images were recorded with the AIDA DVD (Karl Storz, Tuttlingen, Germany).

On day 14, all rats were sacrificed by carbon dioxide inhalation and subjected to necropsy. Cystectomy was performed for histopatho-

logic examination. The bladders were fixed in 10% buffered formalin. After lamination, bladders were embedded in paraffin, and tissue sections of 5 µm were cut and stained with haematoxylin-eosin (HE). Two observers (CAHK, specialized uro-pathologist, and HCA) evaluated the number of tumours, tumour stage (2002 TNM staging system), and tumour grade (2004 WHO/ISUP and WHO 1973 classification). Inflammation in mucosa and submucosa was quantified as no reaction, mild, moderate, or severe reaction. When no tumour was visible on first examination, 2 more sections (each 0.5 mm deeper) were taken, stained, and evaluated.

Results

Animal well-being

Two rats of the initial 36 rats died during anaesthesia (one on day 0, which was excluded from the study, and one on day 5, which was not excluded). A third rat had to be sacrificed prematurely because the humane endpoint was reached. The body weight of this rat decreased more than 10% and haematuria and a decrease in general well-being was observed. On necropsy, the left ureter was dilated, most probably due to oedema around the bladder neck and left ureteral orifice. Histologic examination of the bladder of this rat showed a severe inflammatory reaction in mucosa and submucosa, but no tumour was observed. Mild haematuria after catheterization was observed infrequently. There were no other signs of impaired animal well-being. There were no signs of any toxicity due to the study drug.

Cystoscopy/macroscopy

A large diversity of lesions was seen on cystoscopy on days 5 and 14, ranging from 1 or 2 small lesions to more than 5 small lesions (< 0.5 mm) or even big lesions (> 0.5 mm) per animal. Stone formation was not observed. Figure 1A shows a rat bladder (group 3) with a clearly visible lesion (arrow). The corresponding cystoscopic appearance is

shown in figure 1B, and the histologic aspect of the tumour in figure 1C. However, in 8/12 instances, cystoscopic bladder wall inspection revealed identical defects (i.e., > 5 small lesions or big lesions), which were not confirmed by histology (not shown).

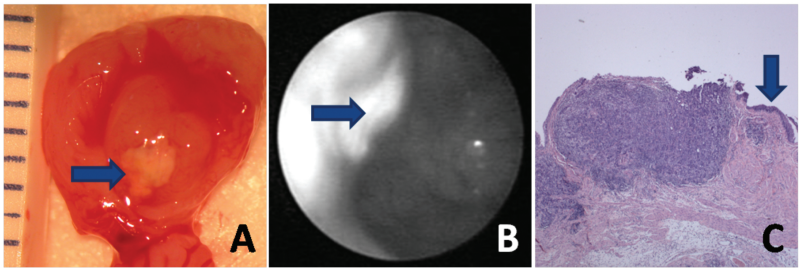

Figure 1A: *Rat bladder after cystectomy opened in longitudinal direction, with clearly visible lesion (arrow) (group 3). The scale is in millimeters.* ***B:*** *Cystoscopic picture of the same lesion (arrow).* ***C:*** *Microscopic picture of the same lesion, a high grade pT2G3 tumour with adjacent normal urothelium (arrow), orginal magnification, ×2.5.*

Histopathology

Normal rat bladder tissue consists of an epithelium of 3 cell layers overlying the submucosa and muscularis. In apaziquone treatment groups, scattered foci of atypical hyperplasia with urothelium of 6 cell layers, moderate nuclear atypia, increased cellularity, increased nuclear/cytoplasmic ratio, apoptosis, and few mitotic figures were observed. The remaining urothelium consisted of 3 cell layers with scattered individual atypical cells with moderate/strong nuclear atypia and low nuclear/cytoplasmic ratio. This histologic appearance is consistent with atypia due to chemotherapeutic instillations.

Inflammatory reactions in mucosa and submucosa were quantified as no reaction, mild, moderate, or severe reaction. In most of the animals, no or only a mild inflammatory reaction was noticed. One rat

in group 1 showed a moderate reaction and 1 rat in group 3 showed a severe reaction; this rat had to be sacrificed prematurely on day 9 (see above).

In table 1, the number of tumours per treatment group are presented. The two rats that died before reaching the end of the experiment, but after they received 2 treatments, were included in the analysis. In case they were excluded, numbers only slightly alter (table 1). In 60% of the animals in the placebo group (group 4), histologic evidence of tumour growth was observed. Histologic examination of the bladders from rats in treatment groups 1, 2, and 3 showed tumours in 0%, 10%, and 20% of rats, respectively. An example of a high grade pT2G3 tumour is shown in figure 1C. Statistical analysis (Fisher's exact test) comparing group 1 with placebo showed that apaziquone treatment was highly effective ($P = 0.022$).

Table 1: *Number of tumours per treatment group.*

Group	Total no. of rats	No. of rats with tumour	No. of rats without tumour	P value vs. placebo
1	10	0	10	0.022
2	10(9)[a]	1	9(8)	0.077 (0.095)
3	10(9)[b]	2	8(7)	0.251 (0.266)
4	5[c]	3	2	Not applicable

[a] *One rat died during anaesthesia on day 5.*

[b] *One rat was sacrificed on day 9 because the humane endpoint was reached, however no tumour was observed. In parenthesis, the numbers when both rats were not included in the analysis.*

[c] *One rat died during anaesthesia on day 0 and was excluded from the study.*

Discussion

Apaziquone, a bioreductive alkylating indoloquinone, was originally developed by The Netherlands Cancer Institute [15]. Based upon its favourable preclinical activity, apaziquone was selected for clinical evaluation and formulated for intravenous administration [4]. However, no clinical responses were documented in the subsequent phase II clinical trials [6,7]. Most probably, the rapid elimination [5] and relatively poor penetration of the drug compromised drug delivery to tumours following systemic administration [16]. Thereafter, apaziquone was tested in the intravesical treatment of urinary bladder cancer, where rapid clearance does not occur [9]. Moreover, the relative stability in urine of the compound during prolonged contact [11] avoided the problems encountered in systemic drug delivery. Finally, its molecular weight (288.3 g/mol) prevents systemic absorption from the bladder. In a dose finding study Puri *et al*. determined that 4 mg/40 mL of apaziquone was well tolerated and could safely be administered in the bladder in patients with NMIBC [9]. Subsequently, Van der Heijden *et al*. performed a phase II marker lesion study on 46 patients with Ta-T1 G1-G2 NMIBC undergoing TURBT [8]. The histologically confirmed complete response 2 to 4 weeks after the last (sixth) instillation was 67% (31/46 patients), and warranted further study. Currently, 3 clinical studies are ongoing: 2 with a single immediate post-TURBT instillation of apaziquone for patients with Ta G1-G2 NMIBC, and 1 with a course of adjuvant apaziquone treatment for patients with high risk and BCG (bacillus Calmette-Guérin)-refractory NMIBC.

During clinical development of apaziquone, buffer composition of the formulation was slightly modified in order to get a better lyophilized product. Although these modifications were not expected to affect the anti-tumour activity of apaziquone, formal evidence was lacking, and it was therefore considered important to examine the activity of different formulations in an orthotopic bladder cancer model.

The efficacy of intravesical chemotherapy depends to a large extent on drug delivery to tumour cells [17]. Inadequate drug delivery is partly the result of dilution by residual urine and continuous urine production during the treatment, and the inability of the drug to penetrate the deeper layers of the bladder. An additional variable is the effect of urinary pH on drug stability. In the present study, the treatment efficacy of the standard apaziquone formulation used in phase I/II clinical trials was compared with the apaziquone formulation with altered buffering capacity used in phase III trials and with the former formulation without propylene glycol in the diluent used to reconstitute apaziquone. Propylene glycol in the diluent was added to improve the solubility of apaziquone, but as the presence of propylene glycol might also influence anti-tumour efficacy by significantly impairing the penetration of apaziquone [12], this formulation was also tested. The placebo solution used in a phase III clinical trial with apaziquone was used as control. All 3 intravesical apaziquone formulations showed excellent anti-tumour activity against the orthotopic rat urothelial cell carcinoma AY-27 compared with placebo. As anticipated, rats treated with the placebo material demonstrated no anti-tumour activity.

The mechanism of action of apaziquone involves reduction by oxidoreductases (particularly DT-diaphorase) and the outcome of therapy is also influenced by oxygen tension. Rat DT-diaphorase (NQO1) is much more efficient at reducing various substrates than human DT-diaphorase and moreover at higher rates [18-20]. This may be a confounding factor of our study.

Intravesical administration of all 3 apaziquone formulations was not associated with any significant acute toxicities. With the exception of 1 animal that had a dilated left ureter, which was most likely not drug-related, the animals showed no signs of impaired animal well-being. Systemic toxicity due to the study drug was not observed. More than 90% of the animals showed no or minimal inflammatory

reaction as judged by histology. Only 2 rats showed a significant inflammatory reaction, which is not unexpected after catheterization and intravesical chemotherapeutic treatment.

Macroscopic tumour growth was assessed by cystoscopy before the second apaziquone bladder instillation and immediately before necropsy. Unfortunately, cystoscopy was of little value to evaluate tumour growth: in only 4/12 bladders, which were scored positive by cystoscopy (i.e., > 5 small lesions or ≥ 1 big lesion) tumours were histologically confirmed. Most probably, these false positive lesions represent reversible reactive oedematous lesions caused by the chemotherapeutic instillation, which also can be seen in the human setting when cystoscopy is done early after an intravesical instillation. This is corroborated by our observation that no discordance between cystoscopy and histology was seen in the placebo group. Unfortunately, cystoscopy is clearly suboptimal for the detection of tumours in this set-up. Most probably, the rather short interval between intravesical instillation and cystoscopy, necessary because of the fast tumour development, hampers reliable cystoscopy. This lack of a reliable method to follow-up tumour development makes it difficult to study treatment outcomes without sacrificing the animals. The histologic examination of the bladder appears to remain the golden standard for the determination of tumour establishment.

Conclusion

Apaziquone showed an excellent anti-tumour activity compared with placebo. The effectiveness of apaziquone in this orthotopic rat bladder tumour model implies the validity of this model, although cystoscopic follow-up of tumours in case of simultaneous intravesical treatments in these rats appears to have limitations. These results also corroborate clinical observations and provide strong preclinical evidence for the use of apaziquone as adjuvant therapy

against non-muscle invasive urothelial carcinoma and support ongoing and future clinical trials.

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References

1. Jemal A, Siegel R, Ward E *et al.*
   Cancer statistics, 2008. CA Cancer J Clin 2008;58:71-96
2. Kirkali Z, Chan T, Manoharan M *et al.*
   Bladder cancer: Epidemiology, staging and grading, and diagnosis. Urology 2005;66:4-34
3. Sylvester RJ, van der Meijden AP, Oosterlinck W *et al.*
   Predicting recurrence and progression in individual patients with stage Ta T1 bladder cancer using EORTC risk tables: A combined analysis of 2596 patients from seven EORTC trials. Eur Urol 2006;49:466-477
4. Hendriks HR, Pizao PE, Berger DP *et al.*
   EO9: A novel bioreductive alkylating indoloquinone with preferential solid tumor activity and lack of bone marrow toxicity in preclinical models. Eur J Cancer 1993;29A:897-906
5. Schellens JH, Planting AS, van Acker BA *et al.*
   Phase I and pharmacologic study of the novel indoloquinone bioreductive alkylating cytotoxic drug EO9. J Natl Cancer Inst 1994;86:906-912
6. Pavlidis N, Hanauske AR, Gamucci T *et al.*
   A randomized phase II study with two schedules of the novel indoloquinone EO9 in non-small-cell lung cancer: A study of the EORTC Early Clinical Studies Group (ECSG). Ann Oncol 1996;7:529-531
7. Dirix LY, Tonnesen F, Cassidy J *et al.*
   EO9 phase II study in advanced breast, gastric, pancreatic, and colorectal carcinoma by the EORTC Early Clinical Studies Group. Eur J Cancer 1996;32A:2019-2022
8. Van der Heijden AG, Moonen PM, Cornel EB *et al.*
   Phase II marker lesion study with intravesical instillation of apaziquone for superficial bladder cancer: Toxicity and marker response. J Urol 2006;176:1349-1353
9. Puri R, Palit V, Loadman PM *et al.*

Phase I/II pilot study of intravesical apaziquone (EO9) for superficial bladder cancer. J Urol 2006;176:1344-1348
10. De Vries JD, Winkelhorst J, Underberg WJM *et al.*
A systematic study on the chemical stability of the novel indoloquinone anti-tumor agent EO9. Int J Pharm 1993;100:181-188
11. Vainchtein LD, Rosing H, Mirejovsky D *et al.*
Stability experiments in human urine with EO9 (apaziquone): A novel anticancer agent for the intravesical treatment of bladder cancer. J Pharm Biomed Anal 2007;43:285-292
12. Abdallah QMA, Gill JH, Lenaz G *et al.*
Analysis of NAD(P)H:quinone oxidoreductase-1 (NQO1), cytochrome P450 oxidoreductase (P450R) and glucose transporter 1 (Glut-1) protein expression suggests a possible role for EO9 in the treatment of invasive bladder cancer. Proc Am Assoc Cancer Res 2006;47:546 [Abstract]
13. Hendricksen K, Molkenboer-Kuenen J, Oosterwijk E *et al.*
Evaluation of an orthotopic rat bladder urothelial cell carcinoma model by cystoscopy. BJU Int 2008;101:889-893
14. Xiao Z, McCallum TJ, Brown KM *et al.*
Characterization of a novel transplantable orthotopic rat bladder transitional cell tumor model. Br J Cancer 1999;81:638-646
15. Oostveen EA and Speckamp WN.
Mitomycin analogs I. Indoloquinones as (potential) *bis*-alkylating agents. Tetrahedron 1987;43:255-262
16. Loadman PM, Phillips RM, Lim LE *et al.*
MC. Pharmacological properties of a new aziridinylbenzoquinone, RH1 (2,5-diaziridinyl-3-(hydroxymethyl)-6-methyl-1,4-benzoquinone), in mice. Biochem Pharmacol 2000;59:831-837
17. Au JL, Badalament RA, Wientjes MG *et al.*
Methods to improve efficacy of intravesical mitomycin C: Results of a randomized phase III trial. J Natl Cancer Inst 2001;93:597-604
18. Beall HD, Mulcahy RT, Siegel D *et al.*
Metabolism of bioreductive antitumor compounds by purified rat and human DT-diaphorases. Cancer Res 1994;54:3196-3201

19. Boland MP, Knox RJ and Roberts JJ.
The differences in kinetics of rat and human DT-diaphorase result in a differential sensitivity of derived cell lines to CB 1954 (5-(aziridin-1-yl)-2,3-dinitrobenzamide). Biochem Pharmacol 1991;41:867-875
20. Gustafson DL, Siegel D, Rastatter JC *et al*.
Kinetics of NAD(P)H: quinone oxidoreductase I (NQO1) inhibition by mitomycin C *in vitro* and *in vivo*. J Pharmacol Exp Ther 2003;305:1079-1086

Chapter 5

Anti-tumour effects of *cis*-urocanic acid on experimental urothelial cell carcinoma of the bladder

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Abstract

Objective

We determined the effect of protodynamic therapy against bladder cancer cells *in vitro* and *in vivo*. We investigated *cis*-urocanic acid (*cis*-UCA) in rat bladder cancer cell cultures and in an orthotopic rat urothelial carcinoma model to assess its safety and anti-proliferative activity.

Animals, materials and methods

The rat bladder cancer cell line AY-27 was exposed to *cis*-urocanic acid (BioCis Pharma, Turku, Finland) at pH 6.5 or 7.4 for 2 hours. Cell viability was measured by colorimetric assay at 24 and 48 hours. For *in vivo* experiments AY-27 cells were instilled into the acid treated bladder of 17 rats. After 4, 7 and 10 days 14 rats were treated intravesically with *cis*-urocanic acid 6% (weight per volume) or vehicle. Rats were sacrificed on day 12 and the bladders were dissected. Immunohistochemical staining was done to assess apoptosis (caspase-3) and cell proliferation (Ki-67) *in vivo*.

Results

Cis-urocanic acid caused dose dependent, pH dependent inhibition of AY-27 cell proliferation, showing the protodynamic action at concentrations of 0.5% and 1%. At higher *cis*-urocanic acid doses complete cell death was observed. All tumours detected in animals treated with vehicle were muscle invasive (stage T2 or greater) but only 43% of tumours were muscle invasive in the *cis*-urocanic acid treated group ($P = 0.049$). There was no difference in the percent of apoptotic or proliferating tumour cells between treatment groups. No signs of toxicity were observed.

Conclusions

Cis-urocanic acid showed direct anti-proliferative activity against rat bladder cancer cells *in vitro* and anti-tumour effects *in vivo*. It may

have therapeutic potential as an intravesical agent for non-muscle invasive bladder cancer.

Introduction

The recommended treatment approach for non-muscle invasive bladder cancer (NMIBC) is complete transurethral resection of the bladder tumour, followed by 1 chemotherapeutic instillation. In tumours at intermediate and high risk for recurrence and/or progression adjuvant intravesical therapy is recommended [1]. However, the clinical effectiveness of current intravesical therapy is limited, and serious local and systemic adverse effects may occur [2]. Thus, new therapeutic options are urgently needed to decrease the toxicity and enhance the efficacy of intravesical treatments of NMIBC.

Protodynamic therapy is a recently introduced concept of cancer treatment involving the inhibition of cancer cell proliferation by intracellular acidification, leading to apoptosis and cell death. *Cis*-Urocanic acid (*cis*-UCA; (2Z)-3-(1*H*-imidazol-4-yl)-2-propenoic acid), a natural amino acid metabolite found in mammalian skin after exposure to ultraviolet radiation, acts as a protodynamic drug capable of transporting protons from the mildly acidic extracellular medium into the tumour cell cytosol [3,4]. *In vitro cis*-UCA treatment showed a direct, permanent anti-proliferative effect in human bladder cancer cell lines in a pH dependent manner and it was superior to 4 other anti-cancer agents studied at therapeutically relevant concentrations to suppress cell proliferation [5]. Since *cis*-UCA is an endogenous compound with no known serious adverse effects, protodynamic therapy with *cis*-UCA is considered a promising new treatment approach for various malignancies. We assessed the safety and anti-tumour activity of repeat intravesical *cis*-UCA administration in the orthotopic Fischer/AY-27 rat bladder urothelial cell carcinoma model *in vivo*.

Animals, materials and methods

The AY-27 urothelial cell carcinoma cell line was originally established as a primary bladder tumour in Fischer F344 rats fed with FANFT (*N*-[4-(5-nitrofuran-2-yl)-1,3-thiazol-2-yl]formamide) [6]. Cells were grown as a monolayer culture in RPMI-1640 medium supplemented with 10% fetal bovine serum (Sigma-Aldrich®), L-glutamine, 100 U/mL penicillin G and 100 µg/mL streptomycin (Invitrogen™) at 37°C in a humidified 95% air/5% CO2 atmosphere. Medium was replenished twice weekly. Cells were passaged using trypsin/ethylenediaminetetraacetic acid (Invitrogen) when confluent.

Cis-UCA was dissolved directly in culture medium to an 8% (weight per volume) concentration. To maintain pH during the experiments medium was supplemented with 25 mM PIPES (Sigma-Aldrich). Thereafter pH was adjusted to pH 6.5 or 7.4 with NaOH. Stock solutions were sterile filtered (0.22 µm) and diluted in the same medium with the same pH for use in assays. Solutions were freshly prepared on the day of use. Culture medium pH was not measured at the end of cell incubation experiments.

AY-27 cells were plated at 10,000 cells per well in 96-well flat bottom microtitration plates. They were incubated at 37°C for 24 hours before treatment in 100 µL culture medium at pH 6.5 and 7.4. Cells were then incubated with 0.5% to 4% *cis*-UCA in medium at pH 6.5 or 7.4 for 2 hours, washed once and incubated for the next 24 and 48 hours in culture medium at the corresponding pH. Cytotoxic activity was determined by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide; Sigma-Aldrich) assay with 30 µL MTT (5 mg/mL) in phosphate-buffered saline added to each well. Blue dye taken up by cells during 4 hours of incubation at 37°C was dissolved in dimethyl sulfoxide (100 µL per well). Optical density at 595 nm was read on an automated 3550 Microplate Reader (Bio-Rad®).

Animal procedures were done after approval by the institutional animal care and use committee of Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands. Procedures were in compliance with national and European regulations. A total of 17 female Fischer F344 rats (Charles River, L'Arbresle, France) weighing 128 to 158 g were housed in individual cages with gold flakes bedding and environmental enrichment in a temperature controlled environment with a 12-hour light/dark cycle, and free access to standard chow and water.

The instillation procedure was done essentially as described by Xiao *et al.* [6] resulting in greater than 80% bladder tumour establishment [7]. Experimental procedures were performed using inhalation anaesthesia. Before each catheterization enrofloxacin (5 to 10 mg/kg) was injected subcutaneously for antibacterial prophylaxis. The rat bladder was catheterized via the urethra with a 16-gauge 1.4 mm plastic intravenous cannula (BD® Biosystems) and drained. The bladder was preconditioned with a 15-second instillation of 0.4 mL 0.1 M HCl and neutralized by 0.4 mL 0.1 M KOH for 15 seconds. The bladder was then drained and conditioned by flushing 3 times with 0.8 mL 0.01 M phosphate buffered saline. Immediately after bladder conditioning AY-27 cells from passage 38 (1.5×10^6 in 0.5 mL medium) harvested within 1 hour before inoculation were instilled in the bladder via a catheter and left indwelling for 60 minutes. Leakage of the cell suspension around the catheter was monitored. Rats were rotated 90 degrees every 15 minutes to facilitate full bladder wall exposure to the cell suspension. The catheter was removed after 60 minutes and the rats were allowed to void spontaneously. Rat well-being was monitored daily with special emphasis on haematuria and weight loss.

Assuming a response rate of 70%, an 85% tumour yield after tumour cell implantation (model evaluation [7]), $\alpha = 0.05$ and a power of 80%, the sample size of the treatment groups was restricted to 7

subjects. Rats were randomly assigned to experimental groups. They were treated intravesically under inhalation anaesthesia with 6% *cis*-UCA or 0.9% NaCl (vehicle) solutions adjusted to pH 5.8 at 4, 7 and 10 days after tumour cell implantation. The solution was retained in the bladder for 60 minutes by clamping the catheter. The rats underwent 90-degree position changes every 15 minutes. After 60 minutes the catheter was removed and the rats were allowed to void spontaneously. On day 12 all 14 rats were sacrificed by CO_2 inhalation and necropsy was done. Cystectomy was performed for histopathological examination. To monitor successful tumour establishment another 3 rats were sacrificed and cystectomy was done 4 days after tumour cell implantation.

Bladders were fixed in buffered 10% formalin. After lamination the bladders were embedded in paraffin. Tissue sections (5 μm) were cut and stained with haematoxylin and eosin. Two observers (specialized uropathologist CAHvdK and HCA) evaluated the number of tumours, tumour stage (2002 TNM staging system), tumour grade (2004 WHO/ISUP and WHO 1973 classifications) and maximum invasion depth. Mucosal and submucosal inflammation was quantified as no reaction, or as mild, moderate or severe reaction.

Immunohistochemical staining was performed to assess cell apoptosis and proliferation *in vivo* using rabbit anti-cleaved caspase-3 antibody (D175, Cell Signaling Technology®) and rabbit anti-Ki-67 monoclonal antibody (SP6, Lab Vision/Neomarkers, Fremont, CA, USA). Briefly, formalin fixed paraffin embedded sections were deparaffinized and subjected to antigen retrieval in 0.01 M sodium citrate buffer, pH 6.0, by boiling in a microwave for 10 minutes. Endogenous peroxidase was quenched by incubation in 0.3% H_2O_2 in 40% methanol for 30 minutes. After pre-incubation in 20% normal goat serum for 30 minutes the primary antibodies were applied at dilutions of 1:1,000 (caspase-3) and 1:200 (Ki-67), and incubated at 4°C overnight. After incubation the slides were treated with biotinylated goat an-

ti-rabbit IgG (H + L) at 1:500 for 30 minutes (Vector Laboratories, Burlingame, CA, USA) and then with a Vectastain® ABC kit according to manufacturer instructions. Sections were developed with 3,3'-diaminobenzidine tetrahydrochloride according to manufacturer instructions (DAB Plus and PowerDAB, ImmunoVision, Brisbane, CA, USA) and counterstained with haematoxylin. Two observers (CAHvdK and HCA) evaluated the slides and estimated the overall percent of apoptotic and proliferative cancer cells.

The statistical significance of differences in viability data *in vitro* was calculated with the 2-way Student t test. Two-tailed P values for differences in tumour invasion and staging were calculated in 2 × 2 contingency tables using the Fisher exact test with $P < 0.05$ considered statistically significant.

Results

Cis-UCA pulse treatment for 2 hours caused dose dependent inhibition of AY-27 cell proliferation (figure 1). At 48 hours after pulse treatment the 0.5% concentration of *cis*-UCA produced a nonsignificant decrease in cell survival of 12% at pH 6.5 and 2% at pH 7.4. The pH 6.5 medium decreased the number of viable cells in the absence of *cis*-UCA by 12% in comparison with the pH 7.4 medium ($P = 0.016$). Incubation with 1% *cis*-UCA resulted in a significant 39% and 29% decrease at pH 6.5 and 7.4 ($P = 0.0036$ and 0.0053, respectively, figure 1). The protodynamic action of *cis*-UCA was shown by the observation that cell viability *in vitro* was decreased more at pH 6.5 than at 7.4 using 0.5% and 1% *cis*-UCA, although the difference was not statistically significant. The 2% to 4% *cis*-UCA doses caused complete cell death at each pH ($P < 0.001$; figure 1), which was already visible 1 day after treatment (data not shown).

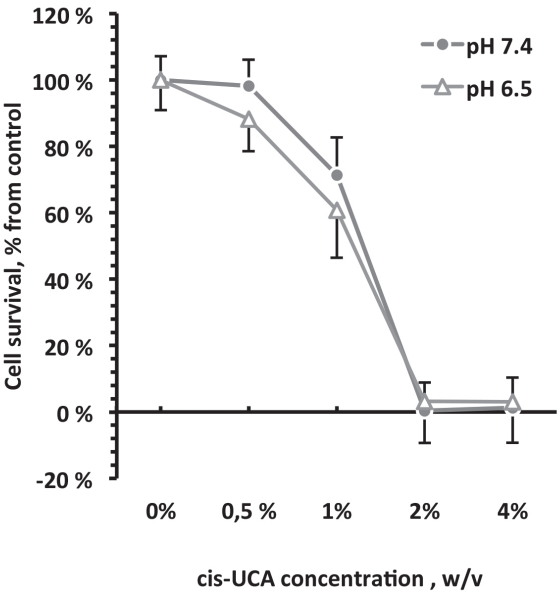

Figure 1: *Mean ± SD percent of viable AY-27 cells in quadruplicate wells according to corresponding pH control after cis-UCA pulse treatment. Cells were incubated with 0.5% to 4% cis-UCA, pH 6.5 or 7.4, for 2 hours, washed and cultured at same pH without cis-UCA for 48 hours. w/v, weight per volume.*

In the orthotopic urothelial cell carcinoma model, in which 14 rats were sacrificed 12 days after tumour cell implantation, a tumour was observed in all except 1 for a total tumour establishment of 93%. In the 13 rats with successful establishment the histologically determined mean maximum tumour invasion depth was 1.65 mm in the vehicle group vs 1.33 mm in the *cis*-UCA group (P = 0.42, figure 2). In all 3 control rats sacrificed 4 days after tumour cell implantation, i.e., before the start of instillation treatments in the remaining animals, histological evidence of tumour establishment was observed with a mean maximum invasion depth of 0.50 mm (figure 2).

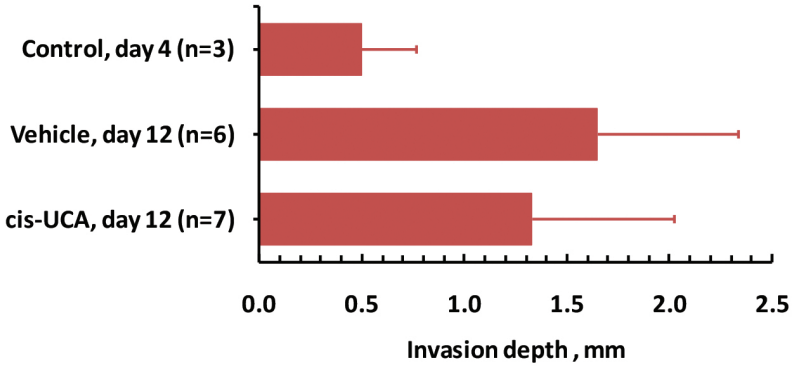

Figure 2: *Mean + SD tumour invasion in F344/AY-27 bladder cancer model in vivo was evaluated histopathologically in bladders dissected 4 (control) and 12 (vehicle and cis-UCA) days after AY-27 tumour cell implantation in bladder of F344 rats with successful tumour establishment.*

For further analysis the observed tumours were divided into 2 groups (Ta-T1 and T2 or greater) according to histologically evaluated tumour stage. In the 3 control rats without treatment the tumours were stages T1a, T1c and T2a, respectively, 4 days after tumour cell implantation. All tumours (100%) observed in the vehicle group were muscle invasive (stage T2 or greater) but only 3 of 7 (43%) in the *cis*-UCA group were evaluated as stage T2 or greater (P = 0.049).

Bladder tissue evaluated for tumour cell apoptosis and proliferation showed 0% to 10% caspase-3 positive tumour cells and about 90% Ki-67 positive tumour cells per rat irrespective of treatment. We noted no gradient of apoptotic cells from superficial to deeper cells.

The body weight of rats in each treatment group increased normally with a slight, temporary decrease 1 day after each anaesthesia. There were no signs of impaired well-being. After catheterization mild haematuria was noted rarely. No sign of toxicity due to the study

drug was observed. Bladder wall inflammatory reaction was anticipated after frequent catheterization. However, no inflammation or only mild/moderate inflammation was noted in most animals in each group.

Discussion

The principal approach to NMIBC management is transurethral bladder tumour resection, followed by intravesical chemotherapy or immunotherapy with bacillus Calmette-Guérin [1]. Since current therapies are only partially effective, often with serious local and systemic adverse effects [2], novel treatment options are required for NMIBC to improve the overall treatment success rate and the toxicity profile.

We present what is to our knowledge the first *in vivo* data on the anti-tumour potential of protodynamic therapy with *cis*-UCA for experimental urothelial cell carcinoma of the bladder. The treatment concept is based on intracellular acidification induced cell death by a weak acid that transports protons into the cytosol of tumour cells in a pH sensitive manner. A clear dose dependent decrease in the viability of cultured AY-27 rat urothelial cell carcinoma cells was observed after pulse treatment with *cis*-UCA for 2 hours. Closely similar responses were reported in 2 human bladder cancer cell lines after a corresponding pulse treatment aimed at modeling the time needed for a clinical intravesical chemotherapy treatment [5].

The effect was more pronounced in the mildly acidic (pH 6.5) conditions than in the neutral/alkaline (pH 7.4) medium, substantiating the protodynamic action. Lack of a major difference in viability between the 2 treatments (pH 6.5 and 7.4) may reflect the fact that the 0.5% and 1% concentrations of *cis*-UCA are high enough to instantly cause a small decrease in the intracellular pH of tumour cells, also in

the pH 7.4 medium [4]. Also, spontaneous acidification of the pH 7.4 medium in the course of a rather long incubation may further decrease the pH difference in the incubation media initially adjusted to pH 6.5 and 7.4 [3], producing a similar protodynamic effect. Mild acidification of medium alone affected cell viability but only to a small extent and similar to results in 2 human bladder cancer cell lines with dissimilar differentiation stages [5]. The dose-response curve was steep, showing that a threshold concentration of *cis*-UCA is needed to enable transport of a sufficient amount of protons under mildly acidic conditions. After this threshold is attained the effect appears irreversible, leading to cell death.

The Fischer/AY-27 rat bladder cancer model is an orthotopic model of urothelial carcinoma [6] with the practical advantage of a fairly short time needed for bladder tumour establishment with a high yield [7]. No complete tumour regression was observed after 3, 1-hour intravesical treatment sessions with 6% *cis*-UCA adjusted to pH 5.8. However, we noted a significant difference in the tumour stage attained after *cis*-UCA treatments. Three control rats sacrificed 4 days after tumour cell implantation already had well established tumours with even muscle invasive disease in 1. Since intravesical treatments were started only 4 days after tumour cell implantation, established tumours may already have been too far advanced for an optimal *cis*-UCA treatment effect. That is, even if superficial cells were effectively treated, invading cells would most probably have remained unscathed.

Immunohistochemical analysis revealed no enhanced tumour cell apoptosis in *cis*-UCA treated animals. However, the slightly reduced tumour thickness suggests that *cis*-UCA treatment resulted in tumour cell kill, most likely at the bladder surface. Our failure to detect more apoptotic tumour cells in *cis*-UCA treated animals was most likely due to the 2-day period between *cis*-UCA treatment and bladder harvest. Also, our *in vitro* experiments suggest that tumour cells

are efficiently killed within 1 to 2 days. This period is probably sufficient to clear the cells in apoptosis induced by *cis*-UCA, as recently reported [3], explaining our observation. This should be studied in future experiments of protodynamic therapy, for instance by evaluation at earlier times after *cis*-UCA treatment.

For effective cancer therapy tumour specific cell kill is the desired outcome to minimize damage to normal tissue. Generally nutritional and metabolic conditions create a major difference between solid tumours and normal tissue, particularly with respect to the extracellular microenvironment in the interstitial space of tumours, which becomes significantly more acidic than in normal tissue. Tumour cells maintain cytosolic pH at neutral or slightly alkaline levels while the extracellular microenvironment is acidified at the same time, usually to around pH 6.7 [8-10]. For anti-tumour activity the protodynamic therapy concept takes advantage of the transmembrane pH gradient in tumours to kill tumour cells. For tumour specificity the concept takes advantage of the inherent difference between extracellular pH in tumours and in normal tissue. As a weak organic acid, *cis*-UCA has favourable acid dissociation properties [11] to achieve enhanced penetration through the plasma membrane [12] to transport and release protons (H+ ions) into the cytosol of tumour cells in a pH dependent manner, resulting in significant intracellular acidification, which triggers apoptosis [3,4]. Since proton transport by this mechanism is not possible in normal tissue in the absence of a transmembrane pH gradient, selective tumour cell kill can be achieved [3-5,8,12].

It is anticipated that an absent protodynamic treatment effect in normal tissue would improve the local and systemic tolerability of *cis*-UCA. As suggested by prior preclinical experience [5], repeat intravesical administration of *cis*-UCA was not associated with any significant (acute) toxicity in this study. The rats showed no sign of impaired well-being and we noted no systemic toxicity due to the study drug.

Conclusion

Cis-UCA showed significant direct anti-proliferative activity against rat bladder cancer cells *in vitro*. Although the number of animals treated was limited, signs of anti-tumour effects were observed and repeat instillations were well tolerated. Thus, *cis*-UCA may have therapeutic potential as an intravesical agent against NMIBC. A phase I dose escalating study was initiated in patients with primary or recurrent NMIBC to test this premise.

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References

1. Babjuk M, Oosterlinck W, Sylvester R *et al.*; European Association of Urology (EAU).
   EAU guidelines on non-muscle-invasive urothelial carcinoma of the bladder. Eur Urol 2008;54:303-314
2. Witjes JA, Palou J, Soloway M *et al.*
   Clinical Practice recommendations for the prevention and management of intravesical therapy-associated adverse events. Eur Urol Suppl 2008;7:667-674
3. Laihia JK, Kallio JP, Taimen P *et al.*
   Protodynamic intracellular acidification by *cis*-urocanic acid promotes apoptosis of melanoma cells *in vitro* and *in vivo*. J Invest Dermatol 2010;130:2431-2439
4. Peuhu E, Kaunisto A, Laihia JK *et al.*
   Molecular targets for the protodynamic action of *cis*-urocanic acid in human bladder carcinoma cells. BMC Cancer 2010;10:521-529
5. Laihia JK, Pylkkänen L, Laato M *et al.*
   Protodynamic therapy for bladder cancer: *in vitro* results of a novel treatment concept. BJU Int 2009;104:1233-1238
6. Xiao Z, McCallum TJ, Brown KM *et al.*
   Characterization of a novel transplantable orthotopic rat bladder transitional cell tumor model. Br J Cancer 1999;81:638-646
7. Hendricksen K, Molkenboer-Kuenen J, Oosterwijk E *et al.*
   Evaluation of an orthotopic rat bladder urothelial cell carcinoma model by cystoscopy. BJU Int 2008;101:889-893
8. Tannock IF and Rotin D.
   Acid pH in tumors and its potential for therapeutic exploitation. Cancer Res 1989;49:4373-4384
9. Gillies RJ, Raghunand N, Karczmar GS *et al.*
   MRI of the tumor microenvironment. J Magn Reson Imaging 2002;16:430-450
10. Kozin SV, Shkarin P and Gerweck LE.

The cell transmembrane pH gradient in tumors enhances cytotoxicity of specific weak acid chemotherapeutics. Cancer Res 2001;61:4740-4743
11. Juusola P, Minkkinen P, Leino L *et al*.
Determination of the dissociation constants of urocanic acid isomers in aqueous solutions. Monatsh Chem 2007;138:951-965
12. Gerweck LE and Seetharaman K.
Cellular pH gradient in tumor versus normal tissue: potential exploitation for the treatment of cancer. Cancer Res 1996;56:1194-1198

Chapter 6

Pharmacokinetics and toxicity of intravesical TMX-101: A preclinical study in pigs

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Abstract

Objective

To study the pharmacokinetic and toxicity profile of intravesically administered TMX-101, with its active ingredient R-837, a synthetic Toll-like receptor (TLR)-7 agonist, in a pig model.

Animals, materials and methods

TLR-7 expression was determined by immunohistochemistry in human and pig bladder tissue. Four groups of six pigs received a 1-h intravesical instillation with R-837 of different formulations. Pharmacokinetic analysis was performed on plasma. Toxicity evaluation included monitoring the well-being of the animals, peripheral blood cell counts, and interleukin-6 and creatinine measurements. Urine was collected for R-837 measurement and dipstick analysis. In total, three pigs per group were sacrificed 24 h post-treatment, and the remaining animals were sacrificed after 1 week. Histopathological examination of the bladder wall was performed.

Results

TLR-7 was homogeneously expressed in human and pig urothelium. R-837 and vehicle were well tolerated without deterioration in animal well-being. Systemic R-837 absorption was low. Mean maximum plasma concentration of R-837 differed depending on the formulation. Post-treatment, plasma levels were negligible at 24 h. Histopathological examination of the bladders did not show significant abnormalities, apart from the intended inflammatory reaction in the R-837 treated groups.

Conclusions

Intravesically administered R-837 in pigs, which showed a similar TLR-7 distribution in bladder tissue as humans, is well tolerated and causes no bladder wall toxicity, and formulations with poloxamer and hydroxypropyl-β-cyclodextrin showed less systemic absorption.

Introduction

The incidence of non-muscle invasive urothelial carcinoma of the bladder is high [1] and the prevalence is even higher as a result of the high recurrence rate after primary transurethral resection. In patients at high risk of tumour recurrence and/or progression to muscle invasive disease, intravesical bacillus Calmette-Guérin (BCG) immunotherapy for at least 1 year is indicated [1]. However, BCG is only partially effective and serious local and systemic side effects may occur [2]. Therefore, the development of new intravesical treatment options to lower tumour recurrence and the progression of non-muscle invasive bladder cancer remains essential.

TMX-101 is an optimized formulation of R-837, 1-(2-methylpropyl)-1*H*-imidazo[4,5-c]quinolin-4-amine, a synthetic low-molecular-weight (240.3 g/mol) immune response modifier and the lead compound of the imidazoquinoline family (Toll-like receptor-7, TLR-7, agonists). Activation of TLR-7 by R-837 leads to an intracellular signalling cascade (MyD88/NF-κB pathway), causing a potent anti-viral and anti-tumour immune response [3]. R-837 is effective and well tolerated as a topical agent (Aldara®; Graceway Pharmaceuticals, LLC, Bristol, TN, USA) for the treatment of various benign and malignant skin lesions [4].

TLR-7 is also expressed in murine and human bladder cancer cell lines and imidazoquinolines have potent direct biological effects on urothelial cell carcinoma cells by decreasing cell viability and inducing apoptosis and cytokine production [5]. The direct effects appear to be the result of c-Myc down-regulation and may synergize with the immunomodulating action of imidazoquinolines [6]. In addition, initial results obtained in an immune competent, orthotopic mouse model suggested the presence of anti-tumour effects *in vivo* [5,6]. Therefore, imidazoquinolines may have therapeutic potential as an intravesical agent for bladder cancer.

To determine whether R-837 may be suitable for bladder cancer therapy, we studied TLR-7 expression in human bladder cancer and normal bladder tissue, followed by an animal study in which three different intravesical formulations of R-837 and a vehicle control were tested for both potential and risks. We studied animal well-being, pharmacokinetic properties, cytokine production and bladder wall histology.

Animals, materials and methods

Detection of TLR-7 expression

In total, 15 formalin-fixed, paraffin-embedded human bladder cancer specimens, six normal bladder specimens and 28 different normal human tissue specimens (other than bladder) were stained for TLR-7 by Mosaic Laboratories, LLC (Lake Forest, CA, USA). In addition, porcine bladder, tonsil, heart, liver, spleen and kidney tissue samples were tested. In short, after antigen retrieval (Mosaic Laboratories, LLC), tissue sections were blocked with protein block (Dako, Carpinteria, CA, USA) for 5 min followed by incubation with anti-TLR-7 antibody (GeneTex, Irvine, CA, USA) diluted in Dako Diluent (Dako) for 30 min. Slides were rinsed in buffer followed by detection using the Envision+ Rabbit HRP detection reagent (Dako) for 30 min. Slides were rinsed and developed with 3,3'-diaminobenzidine (Dako), counterstained with haematoxylin and mounted.

The staining intensity of each specimen was judged relative to the intensity of a control slide containing an adjacent section stained with an irrelevant species- and isotype-matched antibody. Staining of the section labelled with the negative reagent control was considered 'background'. Sections were scored as: 0, no staining relative to background; 1+, weak staining; 2+, moderate staining; 3+ strong staining. Total positive staining (the sum of all staining at 1+, 2+ and 3+) was recorded for each specimen. The *H*-score was calculated based on the summation of the product of percent of cells stained at

each intensity using the equation: (3 × % cells staining at 3+) + (2 × % cells staining at 2+) + (1 × % cells staining at 1+). The *H*-score values were in the range 0-300.

Pig model

Animal procedures were performed according to the protocol approved by the Institutional Animal Care and Use Committee (Radboud University Nijmegen Medical Centre, the Netherlands) and in compliance with national and European regulations.

Female pigs (Dutch Landrace) were used for the present study because the urogenital tract of the pig closely resembles the human urogenital system. The shape of the penis and the preputial diverticulum prevent transurethral catheterization of a male pig. The sows were housed in special swine stainless steel battery cages (Kezkhaff RVS, Ven-Zelderheide, the Netherlands) and fed with universal swine food (Havens Voeders, Maashees, the Netherlands). The pigs were divided into four groups of six animals. Experimental procedures were performed under general anaesthesia. Premedication contained a mixture of 10 mg/kg ketamine and 0.5-1.0 mg/kg midazolam i.m. in one injection. Sedation maintenance was carried out by the same mixture in half the dosage every 45 min. The bladder was emptied before the start of treatment (via 12-F Foley catheter) and 50 mL of the study drug was instilled intravesically. Animals received a solution of R-837 0.5% dissolved in 0.1 M lactic acid (group 1); a solution of R-837 0.5% dissolved in 0.1 M lactic acid, poloxamer 407 16% as emulsifying agent and hydroxypropyl-β-cyclodextrin (HPBCD) 15% as stabilizing agent (group 2); a solution of R-837 0.5% dissolved in 0.1 M lactic acid, poloxamer 407 16% and HPBCD 5% (group 3) or a vehicle control (0.1 M lactic acid) (group 4). The stability of R-837 in all formulations has been checked previously and showed no degradation of the test item. The catheter was clamped and the instillation fluid was retained in the bladder for 60 min, after which the bladder was emptied. The bladder was not rinsed after emptying.

Blood samples for blood cell count and creatinine measurement were collected before instillation and 60 min, 24 h and 1 week (just before cystectomy) after the beginning of instillation. Blood samples for pharmacokinetic analysis and cytokine (interleukin-6, IL-6) measurement were collected before instillation of the study drug and 15, 30, 60, 120, 240 and 480 min after the beginning of drug instillation and also just before cystectomy. Plasma was collected for pharmacokinetic and cytokine analyses, stored at -80°C, and shipped on dry ice for analysis.

The post-treatment evacuated bladder content and the evacuated urine just before necropsy were collected for analysis of R-837 concentration. Urine was frozen immediately and stored in plastic tubes at -80°C, and shipped on dry ice for R-837 determination. Dipstick urinalysis was performed on the evacuated pre- and post-treatment urine and on the urine collected just before necropsy. R-837 concentrations in plasma and urine were determined by CHIMAN s.r.l. (Rottofreno, Italy) by liquid chromatography-mass spectroscopy/mass spectroscopy (LC-MSMS). This method has been validated previously for human urine samples in the range 2-5000 µg/mL R-837 concentrations and was considered adequately accurate (mean accuracy, 104.0%) and precise (mean relative SD, 1.7%). Plasma samples for IL-6 measurement were analyzed by Areta International s.r.l. (Gerenzano, Italy) using the Quantikine Porcine IL-6 (P6000; R&D Systems, Minneapolis, MN, USA) kit.

Body temperature was measured rectally before and 1, 8 and 24 h and 1 week after starting treatment. The well being of the animals was monitored by experienced staff using a selected list of possible signs and symptoms of toxicity before the experiment, just after the instillation and just before cystectomy.

After treatment, three animals per group were sacrificed and cystectomized at 24 h and the remaining animals underwent the same

procedure at 7 days. Material from the bladders was collected and processed for histology: bladder biopsies of 1 cm^2 were taken from dome, trigone, right lateral wall and left lateral wall and transferred into 10% formalin in PBS. Material was embedded in paraffin, sectioned, and stained with haematoxylin and eosin. The slides were evaluated for signs of inflammation and allergic reaction in submucosa and mucosa. Microscopic abnormalities were classified as no reaction, mild, moderate or severe reaction.

Results

TLR-7 expression

TLR-7 expression of the 15 human bladder cancer specimens showed positive staining in all samples, with a mean (SD, range) of 90% (9%, 70-100%). The most intense staining was nuclear membrane/perinuclear and weaker cytoplasmic staining (figure 1A). The mean (SD, range) *H*-score was 127 (23, 90-165). Positive staining was also observed in six normal bladder epithelia specimens, with a mean (SD, range) of 95% (8%, 80 to 100%). Mean (SD, range) *H*-score was 179 (55, 100-230). TLR-7 expression was observed in almost all non-bladder tissues examined (data not shown), most prominently in lymphoid tissue. A lack of staining was observed in heart and smooth muscle. TLR-7 expression in pig tissues (figure 1B) was similar to the corresponding human tissues.

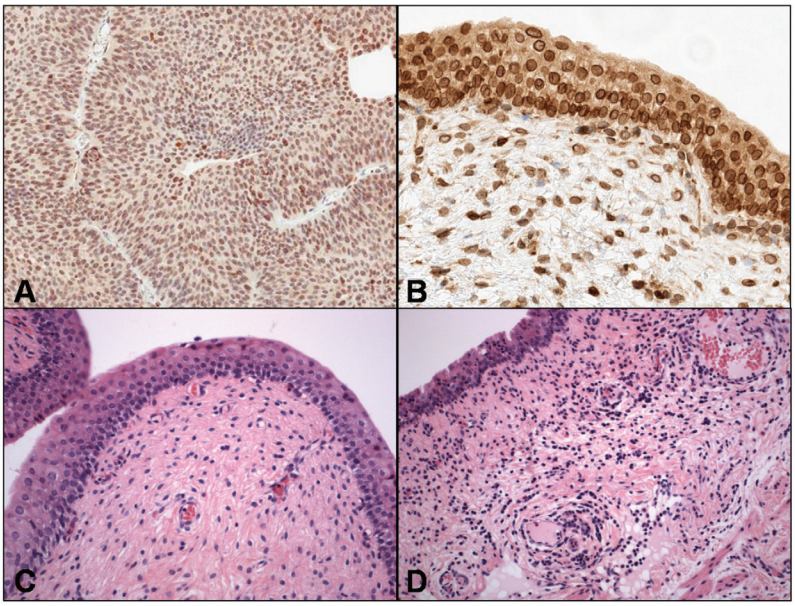

Figure 1 A: *Immunohistochemical staining for Toll-like receptor (TLR)-7 of human non-muscle invasive bladder cancer (original magnification, ×20).* ***B:*** *Immunohistochemical staining for TLR-7 of pig bladder (sub)mucosa (original magnification, ×40).* ***C:*** *Normal (sub)mucosal appearance of a pig bladder (haematoxylin and eosin, original magnification, ×20).* ***D:*** *Moderate predominantly lymphocytic submucosal inflammatory reaction 1 day after instillation of study drug (group 3) (haematoxylin and eosin, original magnification, ×20).*

Pig experiment

A total of 24 pigs with a mean (range) weight of 57.1 (40.0-85.0) kg were divided into four groups of six pigs treated with various formulations of R-837 as a single 60-min intravesical instillation. Throughout the 1-week follow-up period after instillation (three pigs per group), no deterioration of animal well-being was observed. Minor signs of toxicity possibly as a result of the study drug were observed

in four pigs (i.e. low food intake in three pigs, groups 1, 2 and 4; loose stool in one pig, group 1). There were no other signs of impaired animal well-being.

Post-treatment body temperature was not influenced by the instillation of the study drug and was comparable with pre-treatment body temperature for all treatment groups. A slight increase in creatinine levels was observed 1 week post-instillation in groups 1 and 2, without obvious correlation with the treatment modalities.

Haematology values were within the normal range, with the exception of one pig in group 1, which showed abnormal haematology values (haemoglobin concentration, 2.5 mmol/L; haematocrit, 12%; thrombocyte count, 18×10^9/L; leukocyte count, 8.8×10^9/L at the end of the 60-min instillation period). However, 24 h post-treatment almost all haematological values for this animal were within the normal range, except for the thrombocyte count (53×10^9/L), which was within the normal range 1 week post-instillation.

Post-treatment urinalysis (60 min after beginning of instillation) showed high amounts of R-837 (table 1) for all treatment groups, except for the vehicle control group. The amount of R-837 collected in the urine of animals in group 1 was almost twofold higher than that of animals in groups 2 and 3, although this was not significant (ANOVA: $P = 0.096$). After 24 h, R-837 levels were very low (< 5 μg/mL).

*T**able 1:** Total amount of intravesically administered R-837 and the total amount of R-837 in collected post-instillation urine for individual treatment groups.*

Group	Administered total amount R-837 (mg)	End treatment total amount R-837, mg (range) (%)
1	269	219 (23-320) (81.4)
2	258	133 (64-176) (51.5)
3	248	122 (46-219) (49.1)
4	0	0 (-) (Not applicable)

Pharmacokinetic analyses showed only little systemic absorption (figure 2 and table 2). The mean maximum plasma level of group 1 was threefold higher than the mean maximum plasma level of groups 2 and 3 (overall analysis, ANOVA: P = 0.026) resulting in a twofold area under the curve. After 8 h, almost no R-837 (< 2.10 ng/mL) could be detected in any pig plasma.

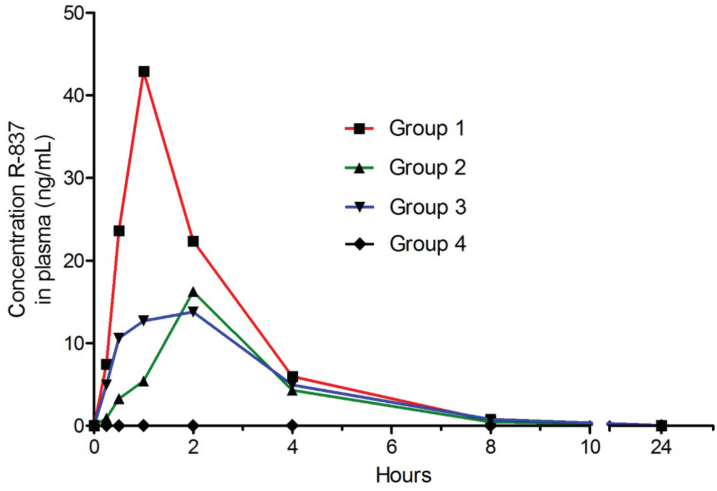

***Figure 2:** Pharmacokinetic plasma parameters of R-837 per treatment group.*

Table 2: *Pharmacokinetic plasma parameters of R-837 per treatment group; mean (SD).*

Group	C_{max} (ng/mL)	AUC (ng*h/mL)	$t_{½}$ (h)
1	45.17 (29.96)	96.75 (50.42)	1.18 (0.12)
2	16.23 (10.22)	45.67 (26.53)	1.58 (1.05)
3	17.00 (6.57)	56.65 (24.94)	1.90 (1.06)
4	0 (0)	0 (0)	Not applicable

C_{max}, Maximum concentration; AUC, area under the curve; $t_{½}$, half-life.

IL-6 cytokine levels were similar in all groups, including the vehicle control group, with maximum IL-6 levels reached 8 h after installation of the study drugs (data not shown).

Macroscopic examination of the resected bladders showed no abnormalities, except for some areas with a haemorrhagical appearance in the pigs sacrificed 24 h post-instillation of TMX-101 (groups 1-3), which was less apparent or absent in the pigs sacrificed after 7 days.

Microscopic examination of the resected bladders showed no difference between the four sampled regions. In most animals from the three treatment groups, a moderate, predominantly lymphocytic submucosal inflammatory reaction was seen 24 h after intravesical instillation (figure 1D), which decreased to mild inflammation in the pigs sacrificed after 7 days. Twenty-four hours post-instillation, lymphocytic vasculitis was observed in three pigs, and was equally divided over the three treatment arms. Moderate myositis was observed in one pig in group 1. Mild reactive atypical bladder epithelium was observed in almost all animals sacrificed after 24 h, which disappeared over time and was not visible in the animals sacrificed after 1 week. Erosion, submucosal oedema and bleeding were mild, and no allergic reaction was observed.

Discussion

Transurethral bladder tumour resection and adjuvant intravesical immunotherapy with BCG is the standard treatment for high-grade non-muscle invasive bladder cancer. However, many patients show a recurrence of disease and the impact on disease progression is only limited [7] or even absent [8]. Moreover, BCG treatment can lead to serious local and systemic side effects [2]. Therefore, novel therapeutic treatment options to improve the overall treatment success rates (possibly with a lower toxicity profile) for non-muscle invasive bladder cancers are urgently needed.

R-837, a member of the imidazoquinoline family, has shown efficacy against many tumour types [9]. The compound binds to TLR-7, inducing the production and secretion of pro-inflammatory cytokines, which consecutively induce a profound tumour-specific cell-mediated immune response, quite similar to the proposed working mechanism of BCG. In addition, R-837 can exert direct apoptotic effects on tumour cells, stimulate TLR-independent gene expression and interfere with adenosine receptor signalling pathways [9].

R-837 is effective and well tolerated as a topical agent for the treatment of various benign and malignant dermatological lesions. Local skin reactions are the most common side effects [10]. R-837 has also been studied as systemic treatment modality: weekly administration of high-dose oral R-837 was studied in a phase I trial in patients with cancer [11]. Dose-limiting side effects were influenza-like symptoms and mild lymphocytopaenia.

Bladder cancer may be an interesting target for R-837 treatment. Intravesical administration of R-837 resembles the topical treatment of skin lesions with direct contact with malignant cells and direct cytotoxicity or apoptosis induced by R-837 independent of an immune response. Moreover, intravesical instillation avoids losses from first-

pass metabolism and allows the therapeutic effect of a drug to be localized at the desirable site with minimal systemic side effects.

Experimental evidence that imidazoquinolines may indeed be appropriate treatment modalities for bladder cancer was provided by Smith *et al*. [5]. These investigators showed that TLR-7 is expressed in murine and human bladder cancer cell lines and that imidazoquinoline has direct biological effects on these cell lines: cell viability was decreased and apoptosis and cytokine production was induced. In addition, initial results obtained in an immune competent, orthotopic mouse model suggested the presence of anti-tumour effects *in vivo* [5,6,12].

To investigate whether the target of R-837, TLR-7, is expressed in human bladder cancer, we evaluated TLR-7 expression in 15 specimens. Positive staining was shown in all samples, with a mean of 90%; however, there was some heterogeneity in intensity, leading to *H*-scores in the range 90-165.

To evaluate the pharmacokinetics and possible toxicity of R-837 installations, experiments in pigs were performed. TLR-7 expression in porcine and human bladder tissue samples was similar, corroborating the validity of the pig model. Moreover, the pig closely resembles the human urogenital tract, providing the most relevant information in a pre-clinical setting. Three different intravesical solutions of R-837 and a vehicle control (lactic acid solution) were studied. None of the tested formulations affected the general well-being of pigs (e.g. as judged by mucosal appearance, behaviour or food/water intake).

Plasma analysis showed only slight systemic absorption of R-837 after bladder instillation, regardless of the formulation used. High amounts of R-837 were recovered in post-instillation urine. Although post-treatment urine R-837 levels in animals treated with R-837 in a

simple lactic acid solution (group 1) were almost twofold higher than in animals treated with R-837 formulations with poloxamer and HP-BCD (groups 2 and 3), this difference was not significant. Nevertheless, the mean maximum plasma level of R-837 in group 1 was threefold higher than in animals from groups 2 and 3 (P = 0.026), most likely as a result of the increased and prolonged bio-adhesiveness of the drug formulations with poloxamer and HPBCD to the bladder wall. However, this effect was short lived: after 8 h, almost no R-837 could be detected in the pig plasma and, after 24 h, R-837 levels in the urine were very low, regardless of R-837 formulation. It is possible that drug formulations 2 and 3 lead to longer, sustained membrane levels of R-837.

Plasma IL-6 levels were similar in all groups, including the vehicle control group, with maximum values being reached 8 h after bladder instillation, most likely as a result of the stress reaction after general anaesthesia and bladder catheterization, rather than immunostimulation by R-837. Moreover, plasma R-837 levels were too low to be considered as physiological relevant.

Histopathological examination of the bladder wall showed the intended inflammatory reaction in the R-837 treated groups. Apart from this intended inflammatory reaction, no significant abnormalities were observed. Only the vasculitis may represent some toxic reaction, albeit this was transient. No vasculitis was observed in the animals sacrificed on day 7. It is not possible to make meaningful intergroup comparisons with these small numbers per group; however, no major difference between the tested R-837 solutions was observed.

The present study specifically examined the toxicity of R-837 in different formulations and is an extension of the study by Hayashi *et al.* [12] who investigated formulations containing R-837 and different ratios of lactic acid, poloxamer and HPBCD in mice. In mice, formu-

lations with poloxamer decreased the systemic absorption of R-837 and significantly reduced systemic and local induction of keratinocyte-derived chemokine. The addition of HPBCD (to enhance stability and solubility) reduced the systemic and local levels of tumour necrosis factor α and keratinocyte-derived chemokine. In the present study, such differences were not observed. However, the effects in mice were only observed at higher dose ranges and, in the present study, R-837 was used at a lower concentration (0,5%) compared to that employed in the mice study (1%). Moreover, the different V/m2 ratio between mouse and pig bladder causes an additional lowering of possible systemic effects. Therefore, the failure to show any differences in toxicity between the groups in the present study was anticipated.

Conclusion

Intravesically administered R-837 in pigs is well tolerated, causes no bladder wall toxicity, and formulations with poloxamer and HPBCD stay longer in the bladder with less systemic absorption. The safety profile of intravesical R-837 appears to be favourable compared to that of current therapies such as BCG. Considering the very similar pharmacokinetic and safety characteristics of the formulations used in groups 2 and 3, further efficacy studies will be initiated with R-837 0.5% in 0.1 M lactic acid, poloxamer 407 16% and HPBCD 5%.

Acknowledgments

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References

1. Babjuk M, Oosterlinck W, Sylvester R *et al.*; European Association of Urology (EAU).
   EAU guidelines on non-muscle-invasive urothelial carcinoma of the bladder. Eur Urol 2008;54:303-314
2. Witjes JA, Palou J, Soloway M *et al.*
   Clinical practice recommendations for the prevention and management of intravesical therapy-associated adverse events. Eur Urol Suppl 2008;7:667-674
3. Hemmi H, Kaisho T, Takeuchi O *et al.*
   Small anti-viral compounds activate immune cells via the TLR7 MyD88-dependent signaling pathway. Nat Immunol 2002;3:196-200
4. Tyring S, Conant M, Marini M *et al.*
   Imiquimod; an international update on therapeutic uses in dermatology. Int J Dermatol 2002;41:810-816
5. Smith EB, Schwartz M, Kawamoto H *et al.*
   Antitumor effects of Imidazoquinolines in urothelial cell carcinoma of the bladder. J Urol 2007;177:2347-2351
6. Liu H, Schwartz MJ, Hwang DH *et al.*
   Tumor growth inhibition by an imidazoquinoline is associated with c-Myc down–regulation in urothelial cell carcinoma. BJU Int 2008;101:894-901
7. Sylvester FJ, Van der Meijden AP and Lamm DL.
   Intravesical bacillus Calmette-Guerin reduces the risk of progression in patients with superficial bladder cancer: a meta-analysis of the published results of randomized clinical trials. J Urol 2002;168:1964-1970
8. Malmström PU, Sylvester RJ, Crawford ED *et al.*
   An individual patient data meta-analysis of the long-term outcome of randomized studies comparing intravesical mitomycin C versus bacillus Calmettte-Guérin for non-muscle-invasive bladder cancer. Eur Urol 2009;56(2):247-256

9. Schön MP and Schön M.
Imiquimod: mode of action. Br J Dermatol 2007;157:8-13
10. Geisse J, Caro I, Lindholm J *et al*.
Imiquimod 5% cream for the treatment of superficial basal cell carcinoma: results from two phase III, randomized, vehicle-controlled studies. J Am Acad Dermatol 2004;50:722-733
11. Witt PL, Ritch PS, Reding D *et al*.
Phase I trial of an oral immunomodulator and interferon inducer in cancer patients. Cancer Res 1993;53:5176-5180
12. Hayashi T, Crain B, Corr M *et al*.
Intravesical Toll-like receptor 7 agonist R-837: optimization of its formulation in an orthotopic mouse model of bladder cancer. Int J Urol 2010;17:483-491

Chapter 7

Summary

Summary

Initial therapy of non-muscle invasive bladder cancer (NMIBC) is transurethral resection, followed by intravesical chemotherapeutic or immunotherapeutic therapy (***chapter 1***). However, a substantial percentage of treated patients still experience tumour recurrences or even progression to muscle invasive bladder cancer because of low compliance with guideline therapy advises, and despite the use of adjuvant intravesical instillations, even when it is used. Moreover, current intravesical therapy is not without toxicity, and serious local and systemic adverse effects may occur. Therefore, improved new intravesical treatment modalities are urgently needed to reduce toxicity and to enhance efficacy. In this thesis the preclinical testing of several potential new intravesical treatment modalities for NMIBC are described.

Photochemical internalization is a novel light-directed drug delivery system, using a photosensitizer specifically localized in the membranes of endosomes. The release of endocytosed drugs into the cytosol of the target cell is triggered by illumination. Results presented in ***chapter 2*** show that meso-tetraphenyl chlorin disulphonate (TPCS2a)-based photochemical internalization significantly potentiates the antiproliferative activity of bleomycin against human and rat urothelial carcinoma cells *in vitro*. The observed treatment effect however was heterogeneous, which is probably a reflection of the heterogeneity of the disease. It is possible that the cellular resistance mechanisms to bleomycin, and the endocytosis and transport mechanisms of bleomycin and/or TPCS2a differ between the various cell lines. Therefore, photochemical internalization may have therapeutic potential as an intravesical strategy against NMIBC, provided predictive patient stratification can be achieved, as discussed in chapter 8.

While *in vitro* bladder tumour models are useful for initial development and evaluation of therapeutic agents and modalities, adequate

animal models are still essential in the preclinical development of new effective and safe therapies. In ***chapter 3*** the problems associated with current *in vivo* animal bladder tumour models are discussed, focusing on the orthotopic syngeneic rat bladder tumour model. Especially the rather short treatment window of the frequently used AY-27/Fischer F344 model hampers its use in the evaluation of new treatments for NMIBC. In the second part of the chapter the development of a potential new orthotopic rat bladder tumour model is described. The initial results of this RBT323/ACI rat model are promising, because after 24 days most of the tumours still seem to be non-muscle invasive providing a reasonably long time window for the administration of study drugs. However, especially tumour cell implantation methods still need to be refined, because all tumours were covered with normal urothelium and prove to be invasive carcinomas (≥ T1) from the start, without any Ta tumours. Also non-invasive *in vivo* diagnostic procedures for the early detection of tumour growth, and follow-up monitoring remains difficult, particularly for early stage non-muscle invasive tumours.

In ***chapter 4*** the results of an efficacy and safety study with different formulations of the relatively new drug apaziquone in the orthotopic Fischer/AY-27 rat bladder cancer model are discussed. Apaziquone showed an excellent anti-tumour activity compared to placebo and there were no signs of any toxicity due to the study drug. The effectiveness of apaziquone in this orthotopic rat bladder tumour model implies the validity of this model, although cystoscopic follow-up of tumours in case of simultaneous intravesical treatments in these rats appears to have limitations, as in 8/12 instances, cystoscopy revealed lesions which were not confirmed by histology. Most probably, these false positive lesions represent reversible reactive oedematous lesions caused by the chemotherapeutic instillation. The results also corroborate clinical observations and provide strong preclinical evidence for the use of apaziquone as adjuvant therapy against non-muscle invasive urothelial carcinoma and support ongoing and future clinical trials.

Another promising novel concept for the treatment of bladder cancer is presented in ***chapter 5***. Protodynamic therapy with *cis*-urocanic acid (*cis*-UCA) comprises the inhibition of cancer cell proliferation by intracellular acidification leading to apoptosis and cell death. Results presented in this chapter show that *cis*-UCA has significant direct anti-proliferative activity against rat bladder cancer cells *in vitro*, as well as inhibitory effects on tumour growth in an orthotopic rat bladder cancer model *in vivo*. Additionally, the study showed that repeated intravesical instillations of *cis*-UCA in rats are well tolerated. Considering the efficacy and good side-effect profile of *cis*-UCA, it may play a role as an intravesical agent in the future.

R-837 is a Toll-like receptor (TLR)-7 agonist and the active ingredient of the promising new intravesical anti-bladder cancer drug TMX-101. Results presented in ***chapter 6*** demonstrate that the distribution of TLR-7 in pig and human bladder tissue is similar and that TLR-7 is highly expressed in human bladder cancer. Furthermore, the results of a porcine pharmacokinetics and toxicity study of three different formulations of TMX-101 are presented. Intravesically administered R-837 in pigs is well tolerated, causes no bladder wall toxicity and formulations with poloxamer and hydroxypropyl-β-cyclodextrin stay longer in the bladder with less systemic absorption.

In conclusion, it is clear that current adjuvant intravesical treatment strategies against NMIBC are suboptimal and need to be improved. This thesis contributes in the continuous search for more effective and less toxic anti-cancer treatments by the preclinical testing of potential emerging intravesical treatment modalities. However, all treatment strategies described in this thesis need more (pre-)clinical studies before they can really contribute in the treatment of patients with bladder cancer.

Samenvatting

De initiële behandeling van niet-spierinvasief blaascarcinoom (NMIBC) is transurethrale resectie, gevolgd door intravesicale chemo- of immunotherapie (***hoofdstuk 1***). Helaas worden de behandelingsadviezen uit de richtlijnen slechts in beperkte mate gevolgd. Bovendien, zelfs wanneer de geadviseerde adjuvante intravesicale instillaties wel worden toegediend, dan ontwikkelt nog steeds een aanzienlijk percentage van de behandelde patiënten een tumorrecidief of zelfs progressie naar spierinvasieve blaaskanker. Daarnaast is het zo dat de huidige intravesicale behandelingen niet zonder toxiciteit zijn en er ernstige lokale en systemische bijwerkingen kunnen optreden. Nieuwe intravesicale behandelingen met minder toxiciteit en verbeterde effectiviteit zijn daarom dringend nodig. In dit proefschrift worden preklinische studies van enkele potentiële nieuwe intravesicale behandelingen voor NMIBC beschreven.

Fotochemische internalisatie is een nieuwe technologie voor lichtgestuurde toediening van geneesmiddelen. Speciale fotosensibilisators accumuleren na toediening specifiek in de membraan van endosomen. De fotosensibilisators worden vervolgens door belichting geactiveerd, waardoor de endosomale membraan wordt vernietigd. Geëndocyteerde geneesmidddelen komen zo vrij in het cytosol en kunnen hun intracellulaire doelwit bereiken. De in ***hoofdstuk 2*** gepresenteerde resultaten laten zien dat fotochemische internalisatie met meso-tetrafenyl chlorine disulfonaat (TPCS2a) de antiproliferatieve activiteit van bleomycine tegen humane en rat urotheelcarcinoom cellen significant versterkt *in vitro*. Het waargenomen effect van de behandeling was echter heterogeen, hetgeen waarschijnlijk een reflectie is van de heterogeniteit van blaaskanker zelf. Het is mogelijk dat de cellulaire afweer mechanismes tegen bleomycine en de endocytose- en/of transportmechanismes van bleomycine en/of TPCS2a verschillen tussen de verschillende cellijnen. Fotochemische internalisatie is daarom een potentiële intravesicale behandelstra-

tegie tegen NMIBC, mits patiënten predictief kunnen worden gestratificeerd, zoals wordt bediscussieerd in hoofdstuk 8.

In vitro blaastumor modellen zijn nuttig voor de initiële ontwikkeling en evaluatie van geneesmiddelen. Echter voor de preklinische ontwikkeling van nieuwe effectieve en veilige behandelingen zijn adequate diermodellen nog steeds essentieel. In ***hoofdstuk 3*** worden de problemen van de huidige *in vivo* blaastumor diermodellen besproken, de focus ligt daarbij op het orthotope syngene rat blaastumor model. Het frequent gebruikte AY-27/Fischer F344 rat model wordt met name beperkt in zijn toepasbaarheid vanwege de relatief korte periode waarin het geschikt is voor het testen van nieuwe behandelingen voor NMIBC. In het tweede deel van het hoofdstuk wordt de ontwikkeling van een potentieel nieuw orthotoop syngeen rat blaastumor model beschreven. De eerste resultaten van dit RBT323/ACI rat model zijn veelbelovend. Na 24 dagen lijkt het grootste deel van de tumoren nog steeds niet-spierinvasief te zijn, waardoor er een redelijk lange periode voor de toediening van studie geneesmiddelen beschikbaar is. Met name de tumorcel implantatie methoden moeten nog worden verfijnd, alle tumoren worden namelijk bedekt met normaal urotheel en bovendien zijn het invasieve carcinomen (≥ T1) vanaf het begin, zonder een enkele Ta tumor. Daarnaast blijven niet-invasieve *in vivo* diagnostische procedures voor de vroege detectie van tumor groei en controle na behandeling moeilijk, met name voor niet-spierinvasieve tumoren in een vroeg stadium.

In ***hoofdstuk 4*** worden de resultaten van een studie naar de effectiviteit en veiligheid van verschillende formuleringen van het relatief nieuwe geneesmiddel apaziquone in het orthotope Fischer/AY-27 rat blaaskanker model besproken. Apaziquone vertoonde een uitstekende anti-tumor activiteit in vergelijking met placebo en er waren geen tekenen van toxiciteit die veroorzaakt werden door het studiegeneesmiddel. De effectiviteit van apaziquone in dit orthotope rat

blaastumor model impliceert de validiteit van het model, hoewel de cystoscopische controle van tumoren in het geval van simultane intravesicale behandelingen beperkingen blijkt te hebben in deze ratten. In 8/12 gevallen toonde cystoscopie namelijk vals positieve laesies, welke meest waarschijnlijk reversibele reactieve oedemateuze laesies vertegenwoordigen, veroorzaakt door de chemotherapeutische instillatie. De resultaten van deze studie bevestigen ook de klinische waarnemingen en leveren sterk preklinisch bewijs voor het gebruik van apaziquone als adjuvante therapie tegen NMIBC en ondersteunen lopende en toekomstige klinische studies.

Een ander veelbelovend nieuw concept voor de behandeling van blaaskanker is beschreven in ***hoofdstuk 5***. Protodynamische behandeling met *cis*-urocaanzuur (*cis*-UCA) remt de proliferatie van kankercellen door intracellulaire aanzuring, hetgeen leidt tot apoptose en celdood. De in dit hoofdstuk gepresenteerde resultaten laten zien dat *cis*-UCA significante directe antiproliferatieve activiteit tegen rat blaaskanker cellen *in vitro* heeft, en bovendien een remmend effect op de tumorgroei in een orthotoop rat blaaskanker model *in vivo*. Daarnaast liet de studie zien dat herhaalde intravesicale instillaties van *cis*-UCA in ratten goed worden getolereerd. Gezien de werkzaamheid en het goede bijwerkingenprofiel van *cis*-UCA, kan het een rol spelen als intravesicaal geneesmiddel in de toekomst.

R-837 is een TLR-7 agonist en het werkzame bestanddeel van het veelbelovende nieuwe intravesicale anti-blaaskanker geneesmiddel TMX-101. De in ***hoofdstuk 6*** gepresenteerde resultaten tonen dat de distributie van TLR-7 in varkens- en humaan blaasweefsel vergelijkbaar is en dat TLR-7 een hoge expressie kent in humaan blaaskankerweefsel. Verder worden de resultaten van een farmacokinetiek- en toxiciteitstudie in varkens gepresenteerd met drie verschillende formuleringen van TMX-101. Intravesicaal toegediend R-837 bij varkens wordt goed getolereerd, het veroorzaakt geen blaaswand toxiciteit

en formuleringen met poloxameer en hydroxypropyl-β-cyclodextrine blijven langer in de blaas met minder systemische absorptie.

Concluderend is het duidelijk dat de huidige adjuvante intravesicale behandelingsstrategieën tegen NMIBC suboptimaal zijn en dus moeten worden verbeterd. Dit proefschrift draagt bij aan de continue zoektocht naar efficiëntere en minder toxische anti-kanker behandelingen door middel van preklinische studies van potentiële intravesicale behandelingen in opkomst. Echter alle behandelingsstrategieën beschreven in dit proefschrift hebben meer (pre-) klinische studies nodig voordat ze echt een bijdrage kunnen leveren aan de behandeling van patiënten met blaaskanker.

Chapter 8

Future perspectives

Future perspectives

Urinary bladder cancer poses a considerable burden on both patients and health care systems. 2.40% of men and women born today will be diagnosed with bladder cancer during their lifetime [1], and, per patient, bladder cancer is the most expensive cancer [2]. The standard initial therapy for non-muscle invasive bladder cancer (NMIBC) is complete macroscopic transurethral resection (TURBT) followed by intravesical therapy [3]. However, compliance with guidelines is low, tumour recurrence is frequent and some tumours are progressing to muscle invasive disease, underscoring the suboptimal clinical outcome of current treatment. Furthermore, current treatment is not without toxicity. This thesis contributes to the continuous scientific pursuit for better therapeutic options through the preclinical testing of new intravesical treatments against NMIBC. Four different treatment modalities are discussed, i.e., photochemical internalization, chemotherapy, protodynamic therapy, and immunotherapy, respectively.

For the preclinical development of new effective and safe intravesical therapies animal models are still essential. However, new regenerative medicine and tissue engineering techniques may offer new possibilities for the replacement of animal models. Janssen *et al.* [4] recently developed an *ex vivo* organoid bladder mucosa model using organotypic culturing for advancing preclinical research of the bladder and to generate alternatives for currently used *in vivo* models. This kind of *in vitro* and *ex vivo* models may support pharmacology and toxicology research to develop new anti-cancer treatments. These models could be used as an alternative for animal experiments and, furthermore, therefore accelerate the preclinical research phase in pharmacological development.

TPCS2a-based photochemical internalization (PCI) of bleomycin may have therapeutic potential as an intravesical strategy against NMIBC. However, the observed treatment effect *in vitro* was heter-

ogeneous, and, as for most anti-cancer treatments, a stratification to predict treatment responses would be highly desirable. A better understanding of tumour biology and pathways critical for tumour genesis may provide personalized treatment opportunities for patients with urothelial cancer, as will be discussed below. Furthermore, preclinical animal studies should be performed to substantiate the value of PCI following our *in vitro* study. For example, a logical next step in the development would be an efficacy study in an orthotopic rat bladder tumour model with 4 treatment groups, i.e., control, bleomycin, TPCS2a-based photodynamic therapy, and TPCS2a-based PCI of bleomycin. A phase I/II study of TPCS2a-based PCI of bleomycin in locally recurrent or advanced/metastatic, (sub) cutaneous malignancies showed no unexpected side effects [5]. Preliminary efficacy data were also very encouraging and a phase II study to evaluate the safety and efficacy of TPCS2a in combination with bleomycin with laser light application in patients with recurrent head and neck squamous cell carcinoma has recently been initiated [6]. Provided that it shows positive results, this trial might pave the way for future trials in NMIBC.

Intravesical chemotherapy with apaziquone is a very promising future treatment modality against NMIBC. Apaziquone showed a complete response of 67% (31/46 patients) in a phase II marker lesion study on 46 patients with TaT1 G1-2 NMIBC undergoing TURBT [7] as already discussed in chapter 4. Hendricksen *et al.* [8] showed that the long-term results of apaziquone treatment are favourable in comparison to the results of other ablative studies: the complete response was followed by a recurrence-free rate of 56.5% at 1-year follow-up, and 49.5% at 2-year follow-up. A phase II study in intermediate- and high-risk NMIBC patients treated with adjuvant intravesical instillations of apaziquone, showed a recurrence in 34.7% of patients after 12 months and 44.9% after 18 months. Only 1 of 53 patients (1.9%) had progression. The instillations were in general well tolerated and the results can be considered promising [9].

Currently, we are waiting for the official publication of the results of two large placebo controlled phase III clinical trials with a single immediate post-TURBT instillation of apaziquone for patients with Ta G1-2 NMIBC [10,11]. Another study with a single immediate post-TURBT instillation of apaziquone for patients with Ta G1-2 NMIBC is still ongoing in Japan and Korea [12]. Spectrum Pharmaceuticals, Inc. (Irvine, CA, USA) already announced in a press release that both phase III trials did not meet their primary endpoint of a statistically significant difference in the rate of tumour recurrence at 2 years between the two arms. However, analysis of the pooled data from both studies showed a statistically significant treatment effect in favour of apaziquone in the primary endpoint of the rate of tumour recurrence at 2 years (P = 0.0174) and in a key secondary endpoint, time to recurrence (P = 0.0076) [13]. However, in both studies, patients at intermediate risk of recurrence with multiple (up to 4) and recurrent tumours were also included. Gudjónsson *et al.* [14] showed that a single, early instillation of epirubicin after TURBT prevents tumour recurrences, mainly in cases of primary, solitary tumours and that such treatment was not beneficial in patients at intermediate- or high-risk of recurrence. Therefore, a subgroup analysis for patients at low-risk of recurrence (i.e., primary, solitary, Ta, G1 tumours) would be interesting in both phase III trials.

Two large international phase III multicenter randomized trials evaluating the efficacy and safety of multiple instillations of intravesical apaziquone versus placebo in patients with low-intermediate risk NMIBC are ongoing with preliminary data collection to be completed by year end 2014 [15,16].

It can be concluded that apaziquone is a very promising intravesical agent for the treatment of NMIBC, its potency and relatively low toxicity have been clearly demonstrated by *in vitro* and *in vivo* preclinical studies, and subsequent phase I and II clinical trials. However, (more) phase III studies are urgently needed before apaziquone

can be positioned as one of the new standards of treatment in patients with NMIBC. The results of our preclinical efficacy and safety study with different formulations of apaziquone corroborate the clinical observations so far, and support ongoing and future clinical trials.

Protodynamic therapy with *cis*-urocanic acid (*cis*-UCA) is another promising novel concept for the treatment of bladder cancer. Considering the efficacy and good side-effect profile of *cis*-UCA observed in our study, it may play a role as an intravesical agent in the future. A phase I study with intravesical *cis*-urocanic acid to evaluate its safety, tolerability and pharmacokinetics in patients with primary or recurrent non-muscle invasive bladder cancer [17] has recently been completed in Finland and we are waiting for the results to be published.

Another potential new intravesical anti-bladder cancer treatment modality is immunotherapy with TMX-101, a Toll-like receptor (TLR)-7 agonist. Our porcine pharmacokinetics and toxicity study of three different intravesical formulations of TMX-101 was followed by a phase I study by Falke *et al*. [18]. This study confirmed the clinical safety of six intravesical instillations with TMX-101. In total, 88 instillations were administered in 16 patients. There was limited dose dependent systemic uptake. The reported side-effects were mild or moderate, mostly local and resolved without intervention. No dose dependant increase in frequency or severity of the adverse events was observed. Currently, a marker lesion study is ongoing in 4 Dutch hospitals to investigate the efficacy of TMX-101 as intravesical treatment for NMIBC patients [19]. Furthermore, there is ongoing preclinical work on a second generation compound, TMX-202. This is a highly specific and potent TLR-7 agonist with a higher molecular mass than TMX-101 and, therefore possible less systemic uptake [20], which might be favourable.

Although this thesis focuses on the development of new intravesical treatment modalities for NMIBC, other aspects are also of great importance to improve future treatment of NMIBC. First of all, to reach future goals in bladder cancer treatment, it is of utmost importance to increase bladder cancer research funding. Carter *et al.* [21] recently revealed that bladder cancer has the lowest research funding per clinical case of all cancers. A considerable mismatch between funding levels and burden was shown. Some cancers are funded at levels far higher than their relative burden suggests, while others (e.g., bladder cancer) appear very much underfunded.

TURBT is a crucial initial procedure in the treatment of NMBC and a complete and correctly performed TURBT is essential to achieve a good prognosis [22]. Training in TURBT is important: it has been shown that surgical experience can improve TURBT results [23]. Moreover, more complete tumour resection can be achieved with new methods of tumour visualization like hexaminolevulinate-guided photodynamic diagnosis (PDD) [24]. If the initial TURBT is optimal, a re-resection could possibly be avoided and the need for a single immediate postoperative instillation reduced. Furthermore, if areas with CIS could be resected completely with the help of PDD or other future techniques, the outcome and prognosis of patients with CIS would hopefully improve.

A major problem in NMIBC management is the suboptimal adherence to evidence based guideline recommendations. Chamie *et al.* [25] retrospectively analyzed 4,545 subjects diagnosed with high-grade NMIBC between 1992 and 2002 who survived at least 2 years without undergoing definitive treatment and found only 1 case of comprehensive compliance. Approximately 42% of physicians have not performed at least 1 cystoscopy, 1 cytology, and 1 instillation of immunotherapy for a single patient within their practice during the initial 2-year period after diagnosis. Physician-attributable variation for individual guideline measures (cystoscopy, 25%; cytology, 59%;

intravesical chemotherapy, 45%; and intravesical immunotherapy, 26%) contributed to this low compliance rate. This study clearly illustrates the urgent need for strategies to improve guideline compliance. First of all, it is critical to identify the barriers for proper guideline compliance. Potential barriers are concerns about side-effects, lack of knowledge about guideline recommendations, and unawareness of the importance of adherence to evidence based guidelines. Effective communication between patient and physician, and education of patient and physician about (the management of) possible side-effects and about the importance of adherence to guidelines are possible strategies to improve guideline compliance. Furthermore, to increase guideline adherence, a guideline should be easy accessible, clear and easy to use. Guidelines should be up to date, however, on the other hand, a frequently changed guideline may hamper guideline adherence. Moreover, differences between e.g., the American Urological Association (AUA) [26] and European Association of Urology (EAU) [3] guidelines, however small, are confusing and a potential obstacle for guideline adherence. Hopefully, the introduction of the simplified risk group stratification in the 2013 EAU guidelines [3], which is more similar to the stratification used in the AUA guidelines, will improve guideline compliance.

Improving the efficacy of currently existing intravesical treatments is another option to improve NMIBC treatment. Enhancing drug delivery to tumour cells could be achieved by controlling physiologic variables and by increasing the dose. Relatively simple measures as adapting urinary pH, decreasing urinary excretion, and buffering the intravesical solution can already enhance efficacy [27].

The efficacy of mitomycin C can also be enhanced using microwave-induced hyperthermia (thermochemotherapy; Synergo) or electromotive drug administration (EMDA). These device-assisted therapies facilitate chemotherapeutic drug transport into the urothelium and have the potential to improve treatment outcome.

Hyperthermia is thought to increase the penetration of mitomycine C into the urothelium due to increased cellular membrane permeability and/or modified blood perfusion. In a recent systematic review of thermochemotherapy [28], it was reported that thermochemotherapy offered a 59% relative reduction in NMIBC recurrence compared with mitomycin C alone, with a bladder preservation rate of 87.6%. Currently, there are 2 ongoing phase III trials with thermochemotherapy. In 2002, a multicenter phase III study was started to compare 1 year of BCG with 1 year of thermochemotherapy with mitomycin C. A total of 300 high-risk patients will be randomized. Primary outcome measures are recurrence-free survival, time to complete response (for CIS), and progression rate [29]. Another phase III study compares thermochemotherapy with mitomycin C to BCG or standard therapy after failure of intravesical therapy. A total of 242 patients will be randomized to thermochemotherapy and a second induction course of BCG with subsequent maintenance for patients that previously failed induction BCG or standard therapy for patients that previously failed maintenance BCG. Standard therapy is defined by the treating centers. Primary outcome measures are disease-free survival and complete response rates (for CIS) [30]. Most clinical thermochemotherapy trials were performed with mitomycin C. However, Van der Heijden *et al.* [31] investigated the *in vitro* effect of hyperthermia on 4 chemotherapeutic agents and found that apaziquone was the most potent drug. So future trials investigating the effect of thermochemotherapy with apaziquone would also be very interesting.

EMDA is mainly based on iontophoresis which represents the electrokinetic migration of ionic molecules in an electric field. A randomized prospective study of EMDA mitomycin C versus passive mitomycin C versus standard BCG was conducted in 108 BCG-naïve patients with high-risk NMIBC and showed that EMDA mitomycin C is equal to BCG in terms of recurrence rate and is significantly superior to passive standard mitomycin C in this patient group [32,33].

Recently, Di Stasi *et al.* [34] showed that intravesical EMDA mitomycin C before TURBT reduces recurrence rates and enhances the disease-free interval compared with passive intravesical mitomycin C after TURBT and TURBT alone. Currently, device assisted treatment modalities are still considered experimental, and predominantly used as bladder preservation strategy in patients failing on BCG and unfit or unwilling to undergo cystectomy. More phase III trials with sufficient follow up are needed to elucidate their role in standard NMIBC therapy.

Urothelial carcinoma of the bladder is a heterogeneous disease. It ranges from non-muscle invasive to muscle-invasive to advanced disease. Each clinical state is associated with a unique tumour biology, prognosis, and approach to treatment. Non-muscle invasive bladder cancer is further stratified in a low, intermediate and high risk group based on clinical and pathological factors [3]. Ideally, in the future, patients can be offered a personalized treatment of NMIBC reflecting the individual nature of each tumour, intratumour heterogeneity [35], and (epi)genomic background of the patient. Genetic and molecular profiling of tissue and/or urine may improve risk assignment and direct novel targeted treatment strategies to increase efficacy and minimize toxicity. Hopefully, future advances in molecular biology may accurately predict which treatment will be successful and which tumours will not respond to intravesical therapy. For patients predicted as non-responders to conservative intravesical treatment an early radical cystectomy can be offered, preventing unnecessary burden of intravesical treatment and moreover preventing possible disease progression in time. For example, Agundez *et al.* [36] measured retrospectively the methylation status of 25 tumour suppressor genes in 91 patients with T1G3 tumours undergoing BCG treatment. It was shown that the combination of *MSH6* and *THBS1* provided the most significant predictive assessment for progression (P = 0.004). The promising clinical relevance of tumour suppressor genes methylation assessment as BCG predictive biomarkers warrants further

elucidation in prospective trials to evaluate whether they could suggest therapeutic decisions. In general, an increased armamentarium of treatment modalities aiming at different targets, as described here, might be valuable to reach the goal of personalized treatment of NMIBC patients leading to reduced recurrence and progression rates and better quality of life.

References

1. Howlader N, Noone AM, Krapcho M *et al.* (eds).
   SEER Cancer Statistics Review, 1975-2010, National Cancer Institute. Bethesda (MD), Available from: http://seer.cancer.gov/csr/1975_2010
2. Botteman MF, Pashos CL, Redaelli A *et al.*
   The health economics of bladder cancer. A comprehensive review of the published literature. Pharmacoeconomics 2003;21:1315-1330
3. Babjuk M, Burger M, Zigeuner R *et al.*
   EAU Guidelines on non-muscle-invasive urothelial carcinoma of the bladder: Update 2013. Eur Urol 2013;64:639-653
4. Janssen DAW, Geutjes PJ, Odenthal J *et al.*
   A new and straight forward ex vivo organoid bladder mucosa model for preclinical research. J Urol 2013;190:341-349
5. Forster M, Sultan A, Jerjes W *et al.*
   First-in-man phase I study of TPCS2a-based photochemical internalisation (PCI) of bleomycin in locally recurrent or advanced/metastatic, cutaneous or subcutaneous malignancies. Ann oncol 2012;23:347-347
6. ClinicalTrials.gov [Internet].
   Bethesda (MD): National Library of Medicine (US). 2000 Feb 29. Identifier NCT01606566, An open-label, single arm, multi-centre phase II study to evaluate safety and efficacy of PC-A11 in patients with recurrent non-metastatic head and neck squamous cell carcinoma unsuitable for surgery and radiotherapy; 2012 May 21 [cited 2013 May 23]; Available from: http://clinicaltrials.gov/ct/show/ NCT01606566?order=1
7. Van der Heijden AG, Moonen PM, Cornel EB *et al.*
   Phase II marker lesion study with intravesical instillation of apaziquone for superficial bladder cancer: toxicity and marker response. J Urol 2006;176:1349-1353
8. Hendricksen K, Van der Heijden AG, Cornel EB *et al.*
   Two-year follow-up of the phase II marker lesion study of intra-

vesical apaziquone for patients with non-muscle invasive bladder cancer. World J Urol 2009;27:337-342
9. Hendricksen K, Cornel EB, De Reijke TM *et al*.
Phase 2 study of adjuvant intravesical instillations of apaziquone in high-risk non-muscle invasive bladder cancer. J Urol. 2012;187:1195-1199
10. ClinicalTrials.gov [Internet].
Bethesda (MD): National Library of Medicine (US). 2000 Feb 29. Identifier NCT00461591, A multicenter, randomized, placebo-controlled, double-blind, phase 3 trial of single dose intravesical EOquin® as a surgical adjuvant instilled in early post operative period in patients undergoing transurethral resection for noninvasive bladder cancer; 2007 Apr 16 [cited 2013 May 23]; Available from: http://clinicaltrials.gov/ct/show/NCT00461591?order=1
11. ClinicalTrials.gov [Internet].
Bethesda (MD): National Library of Medicine (US). 2000 Feb 29. Identifier NCT00598806, A multicenter, randomized, placebo-controlled, double-blind phase 3 trial of single-dose intravesical EOquin® as a surgical adjuvant instilled in the early postoperative period in patients undergoing transurethral resection for noninvasive bladder cancer; 2008 Jan 11 [cited 2013 May 23]; Available from: http://clinicaltrials.gov/ct/show/NCT00598806?order=1
12. ClinicalTrials.gov [Internet].
Bethesda (MD): National Library of Medicine (US). 2000 Feb 29. Identifier NCT01475266, Randomized, placebo-controlled, double-blinded study of single immediate instillation of EO9 after TURBT in patients with NMIBC; 2011 Nov 16 [cited 2013 May 23]; Available from: http://clinicaltrials.gov/ct/show/NCT01475266?order=1
13. Spectrum Pharmaceuticals, Inc. Irvine (CA).
Spectrum Pharmaceuticals announces results of apaziquone phase 3 clinical trials, 2012 April 5. [cited 2013 July 26]; Available from: http://investor.spectrumpharm.com/releasedetail.cfm?ReleaseID=662042

14. Gudjonsson S, Adell L, Merdasa F *et al.*
Should all patients with non-muscle-invasive bladder cancer receive early intravesical chemotherapy after transurethral resection? The results of a prospective randomized multicentre study. Eur Urol 2009;55:773-780
15. ClinicalTrials.gov [Internet].
Bethesda (MD): National Library of Medicine (US). 2000 Feb 29. Identifier NCT01410565, A phase 3 international, multicenter, double-blind, placebo-controlled, randomized trial evaluating the efficacy and safety of multiple instillations of intravesical apaziquone vs. placebo in patients with low-intermediate risk non-muscle invasive bladder cancer (NMIBC); 2011 Aug 1 [cited 2013 May 23]; Available from: http://clinicaltrials.gov/ct/show/NCT01410565?order=1
16. ClinicalTrials.gov [Internet].
Bethesda (MD): National Library of Medicine (US). 2000 Feb 29. Identifier NCT01469221, A phase 3 international, double-bind trial evaluating efficacy and safety of multiple instillations of intravesical apaziquone vs. placebo in patients with low-intermediate risk non-muscle invasive bladder cancer (NMIBC); 2011 Nov 8 [cited 2013 May 23]; Available from: http://clinicaltrials.gov/ct/show/NCT01469221?order=1
17. ClinicalTrials.gov [Internet].
Bethesda (MD): National Library of Medicine (US). 2000 Feb 29. Identifier NCT01458847, A single dose, dose-escalating, phase I study to evaluate safety, tolerability and pharmacokinetics of intravesical *cis*-urocanic acid (*cis*-UCA) in patients with primary or recurrent non-muscle invasive bladder cancer; 2011 Oct 4 [cited 2013 May 23]; Available from: http://clinicaltrials.gov/ct/show/NCT01458847?order=1
18. Falke J, Lammers RJM, Arentsen HC *et al.*
Results of a phase 1 dose escalation study of intravesical TMX-101 in patients with non-muscle-invasive bladder cancer. J Urol 2013;189:2077-2082

19. Registration EudraCT number 2009-014757-33
20. Falke J, Arentsen HC, Hulsbergen-Van De Kaa CA *et al.*
Pharmacokinetics and toxicity of R-837 and TMX-202 after intravesical and intravenous administration: A pre-clinical study in naive rats. Eur Urol Suppl 2012;11:e665
21. Carter AJR and Nguyen CN.
A comparison of cancer burden and research spending reveals discrepancies in the distribution of research funding. BMC Public Health 2012;12:526-537
22. Brausi M, Collette L, Kurth K *et al.*, EORTC Genito-Urinary Tract Cancer Collaborative Group.
Variability in the recurrence rate at first follow-up cystoscopy after TUR in stage Ta T1 transitional cell carcinoma of the bladder: a combined analysis of seven EORTC studies. Eur Urol 2002;41:523-31
23. Mariappan P, Finney SM, Head E *et al.*
Good quality white-light transurethral resection of bladder tumours (GQ-WLTURBT) with experienced surgeons performing complete resections and obtaining detrusor muscle reduces early recurrence in new non-muscle-invasive bladder cancer: validation across time and place and recommendation for benchmarking. BJU Int 2012;109:1666-1673
24. Burger M, Grossmann HB, Droller M *et al.*
Photodynamic diagnosis of non-muscle-invasive bladder cancer with hexaminolevulinate cystoscopy: a meta-analysis of detection and recurrence based on raw data. Eur Urol 2013;64:846-854
25. Chamie K, Saigal CS, Lai J *et al.*
The Urologic Diseases in America Project. Compliance with guidelines for patients with bladder cancer: variation in the delivery of care. Cancer 2011;117: 5392-5401
26. Hall MC, Chang SS, Dalbagni G *et al.*
Guideline for the management of nonmuscle invasive bladder cancer (stages Ta, T1, and Tis): 2007 update. J Urol 2007;178:2314-2330

27. Au JL, Badalament RA, Wientjes MG *et al.*
Methods to improve efficacy of intravesical mitomycin C: results of a randomized phase III trial. J Natl Cancer Inst 2001;93:597-604
28. Lammers RJM, Witjes JA, Inman BA *et al.*
The role of a combined regimen with intravesical chemotherapy and hyperthermia in the management of non-muscle-invasive bladder cancer: a systematic review. Eur Urol 2011;60:81-93
29. ClinicalTrials.gov [Internet].
Bethesda (MD): National Library of Medicine (US). 2000 Feb 29. Identifier NCT00384891, A randomized controlled study comparing adjuvant hyperthermia treatment in conjunction with mitomycin C versus BCG immunotherapy (BCG) adjuvant treatment in patients with superficial transitional cell carcinoma of the bladder (STCCB); 2006 Oct 5 [cited 2013 May 23]; Available from: http://www.clinicaltrials.gov/ct2/show/NCT00384891?order=1
30. ClinicalTrials.gov [Internet].
Bethesda (MD): National Library of Medicine (US). 2000 Feb 29. Identifier NCT01094964, HYMN: a randomized controlled phase III trial comparing hyperthermia plus mitomycin to a second course of bacillus Calmette-Guerin or standard therapy in patients with recurrence of nonmuscle invasive bladder cancer following induction or maintenance bacillus Calmette-Guerin therapy; 2010 March 26 [cited May 23]; Available from: http://www.clinicaltrials.gov/ct2/show/NCT01094964?order=1
31. Van der Heijden AG, Verhaegh G, Jansen CF *et al.*
Effect of hyperthermia on the cytotoxicity of 4 chemotherapeutic agents currently used for the treatment of transitional cell carcinoma of the bladder: an *in vitro* study. J Urol 2005;173:1375-1380
32. Di Stasi SM, Giannantoni A, Stephen RL *et al.*
Intravesical electromotive mitomycin C versus passive transport mitomycin C for high risk superficial bladder cancer: a prospective randomized study. J Urol 2003;170:777-782
33. Di Stasi SM and Riedl C.
Updates in intravesical electromotive drug administration of mi-

tomycin-C for non-muscle invasive bladder cancer. World J Urol 2009;27:325-330
34. Di Stasi SM, Valenti M, Verri C *et al.*
Electromotive instillation of mitomycin immediately before transurethral resection for patients with primary urothelial non-muscle invasive bladder cancer: a randomized controlled trial. Lancet Oncol 2011;12:871-879
35. Gerlinger M, Rowan AJ, Horswell S *et al.*
Intratumor heterogeneity and branched evolution revealed by multiregion sequencing. N Engl J Med 2012;366:883-892
36. Agundez M, Grau L, Palou J *et al.*
Evaluation of the methylation status of tumour suppressor genes for predicting bacillus Calmette-Guérin response in patients with T1G3 high-risk bladder tumours. Eur Urol 2011;60:131-140

Appendix 1

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Acknowledgements (Dankwoord)

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Appendix 2

Bibliography

Bibliography

Arentsen HC, de la Rosette JJMCH, de Reijke TM, Langbein S.
Fluorescence *in situ* hybridization: a multitarget approach in diagnosis and management of urothelial cancer. Expert Rev Mol Diagn. 2007;7:11-19

Arentsen HC, Langbein S, de la Rosette JJMCH, Pelger RCM, Bos SD, Fernandes JG, de Reijke TM.
The Nuclear Matrix Protein 22 (NMP22®) BladderChek® Test for the detection of bladder cancer. Urol Pol. 2008;61:5-8

Arentsen HC, Hendricksen K, Oosterwijk E, Witjes JA.
Experimental rat bladder urothelial cell carcinoma models. World J Urol. 2009;27:313-317

Arentsen HC, Hendricksen K, Hulsbergen-van de Kaa CA, Reddy G, Oosterwijk E, Witjes JA.
The orthotopic Fischer/AY-27 rat bladder urothelial cell carcinoma model to test the efficacy of different Apaziquone formulations. Urol Oncol. 2012;30:64-68

Witjes JA, Palou J, Jacqmin D, Sofras F, Malmström PU, Riedl C, Jocham D, Conti G, Montorsi F, **Arentsen HC**, Zaak D, Mostafid AH, Babjuk M.
Hexylaminolevulinate (Hexvix®)-guided fluorescence cystoscopy in the diagnosis and follow up of patients with non-muscle invasive bladder cancer: review of the evidence and recommendations from a European expert panel. Eur Urol. 2010;57:607-614

Arentsen HC, Hulsbergen-van de Kaa CA, Jansen CFJ, Maj R, Leoni LM, Oosterwijk E, Witjes JA.
Pharmacokinetics and toxicity of intravesical TMX-101: A preclinical study in pigs. BJU Int. 2011;108:1210-1214

Arentsen HC, Jansen CFJ, Hulsbergen-van de Kaa CA, Laihia JK, Pylkkänen L, Leino L, Oosterwijk E, Witjes JA.
Antitumor effects of *cis*-urocanic acid on experimental urothelial cell carcinoma of the bladder. J Urol. 2012;187:1445-1449

Hendricksen K, Cornel EB, de Reijke TM, **Arentsen HC**, Chawla S, Witjes JA.
Phase 2 study of adjuvant intravesical instillations of apaziquone in high-risk non-muscle invasive bladder cancer. J Urol. 2012;187:1195-1199

Falke J, Lammers RJM, **Arentsen HC**, Ravic M, Pozzi R, Cornel EB, Vergunst H, de Reijke TM, Witjes JA.
Results of a phase 1 dose escalation study of intravesical TMX-101 in patients with non-muscle-invasive bladder cancer. J Urol. 2013;189:2077-2082

Arentsen HC, Falke J, Høgset A, Oosterwijk E, Witjes JA.
The effect of photochemical internalization of bleomycin in the treatment of urothelial carcinoma of the bladder: an *in vitro* study. Urol Oncol. 2014;32:49.e1-49.e6

Appendix 3

Curriculum Vitae

Harm Christiaan Arentsen

De auteur van dit proefschrift werd op 17 april 1980 geboren in de Achterhoekse gemeente Gendringen, waar hij opgroeide samen met zijn 3 jongere broers. Op 1 juli 1998 behaalde hij het Atheneum diploma aan het Isala College te Silvolde. In datzelfde jaar werd begonnen met de studie geneeskunde aan de Vrije Universiteit Brussel in België. Naast studeren, leidde hij een actief studentikoos leven, waarin hij onder andere een jaar praeses was van Hoogstudentenclub 'Boves Luci'. Het laatste, zogenaamde pre-specialisatie jaar van de opleiding, bestond uit een klinische en wetenschappelijke stage op de afdeling urologie van het Universitair Ziekenhuis Brussel (Prof. F. Keuppens) en uit klinische stages op de afdelingen urologie van het ZNA Middelheim te Antwerpen (P. Van Erps) en het Universitair Medisch Centrum St Radboud te Nijmegen (Prof. dr. F.M.J. Debruyne). De academische graad van Arts (Master) werd behaald 'met onderscheiding' op 8 juli 2005.

Van augustus 2005 tot en met december 2006 werkte hij op de afdeling urologie van het Academisch Medisch Centrum te Amsterdam (Prof. dr. J.J.M.C.H. de la Rosette), aanvankelijk als ANIOS (arts niet in opleiding tot specialist) en vanaf 2006 als arts-onderzoeker. In januari 2007 begon hij als ANIOS op de afdeling urologie van het Jeroen Bosch Ziekenhuis te 's-Hertogenbosch (dr. H.P. Beerlage).

Op 1 december 2007 startte hij met zijn promotieonderzoek op de afdeling urologie van het Universitair Medisch Centrum St Radboud te Nijmegen (Prof. dr. J.A. Witjes). De resultaten van dit promotieonderzoek staan beschreven in dit proefschrift. De afzonderlijke studies werden gepubliceerd in vooraanstaande internationale urologische tijdschriften en gepresenteerd op (inter)nationale urologische congressen.

In het kader van de opleiding tot uroloog werd op 1 januari 2010 begonnen met de vooropleiding chirurgie in het Rijnstate ziekenhuis te Arnhem (dr. M.M.P.J. Reijnen). In januari 2012 werd vervolgens gestart met de specialisatie tot uroloog op de afdeling urologie van het Canisius-Wilhelmina Ziekenhuis te Nijmegen (dr. H. Vergunst). Het academische gedeelte van de opleiding zal daarna (januari 2014) plaatsvinden op de afdeling urologie van het Universitair Medisch Centrum St Radboud te Nijmegen (Prof. dr. J.A. Witjes).

In 2012 ontving hij de Prof. dr. W.A. Moonenprijs van de Nederlandse Vereniging voor Urologie voor zijn onderzoek naar de farmacokinetiek en toxiciteit van het anti-blaaskanker middel TMX-101.

Harm Christiaan Arentsen

The author of this thesis was born on April 17, 1980 in the municipality of Gendringen in the Dutch region of the 'Achterhoek', and was raised together with his 3 younger brothers. On July 1, 1998 he graduated from the Isala College in Silvolde, the Netherlands, with his Atheneum diploma. In the same year he started studying medicine at the Free University of Brussels, Belgium. Besides studying, he led an active student life and for one year he was president of the student society 'Boves Luci'. During the pre-residency year of his medical training he conducted a clinical and a scientific internship at the department of urology of the University Hospital Brussels in Brussels, Belgium (Prof. F. Keuppens, MD) and clinical internships at the departments of urology of the ZNA Middelheim Hospital in Antwerp, Belgium (P. Van Erps, MD) and the Radboud University Nijmegen Medical Centre in Nijmegen, the Netherlands (Prof. F.M.J. Debruyne, MD, PhD). He obtained his medical degree cum laude on July 8, 2005.

As of August 2005 until December 2006 he worked at the department of urology of the Academic Medical Centre in Amsterdam, the Netherlands (Prof. J.J.M.C.H. de la Rosette, MD, PhD), first as a resident (pre-training) and as of 2006 as a clinical researcher. In January 2007 he started working as a resident (pre-training) at the department of urology of the Jeroen Bosch Hospital in 's-Hertogenbosch, the Netherlands (H.P. Beerlage, MD, PhD).

As of December 1, 2007 until December 31, 2009 he worked on the present dissertation at the department of urology of the Radboud University Nijmegen Medical Centre in Nijmegen, the Netherlands (Prof. J.A. Witjes, MD, PhD). The individual studies were published in prominent international urological journals and presented at national and international urological congresses.

Within the framework of his formal training in urology, he started his residency in general surgery at the Rijnstate Hospital in Arnhem, the Netherlands (M.M.P.J. Reijnen, MD, PhD) on January 1, 2010. Subsequently, he started with his urological training in January 2012 at the department of urology in the Canisius-Wilhelmina Hospital in Nijmegen, the Netherlands (H. Vergunst, MD, PhD). In January 2014 he will return to the department of urology of the Radboud University Nijmegen Medical Centre in Nijmegen, the Netherlands (Prof. J.A. Witjes, MD, PhD) for the academic part of his urological training.

In 2012 he was awarded with the Prof. dr. W.A. Moonen award of the Dutch Urological Association for his research on the pharmacokinetics and toxicity of the anti-bladder cancer agent TMX-101.